UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

MODULATING THE BONE MARROW MICROENVIRONMENT:

THE ROLE OF DLL4:NOTCH SIGNALING AND HYPOXIA

Leonor Remédio

Doutoramento em Biologia
Especialidade de Biologia Celular

2013

UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

MODULATING THE BONE MARROW MICROENVIRONMENT:

THE ROLE OF DLL4:NOTCH SIGNALING AND HYPOXIA

Leonor Remédio

Tese orientada pelo Professor Doutor Sérgio Dias e pela Professora Doutora Gabriela Rodrigues, especialmente elaborada para a obtenção do grau de doutor em Biologia, especialidade de Biologia Celular.

2013

This work was supported by the fellowship SFRH / BD / 48497 / 2008 from the Foundation for Science and Technology, Portugal.

The chapters of results presented in this dissertation include one published scientific article, and two others in preparation for later publication in indexed international scientific journals. Given that these works were accomplished in collaboration with other investigators, and according to the disposed in n^{er} 1 of the Article 40th of the Regulation of the Postgraduate Studies of the University of Lisbon, published in Diário da República - n^{er} 153, II Series of July 5th 2003, I hereby clarify that I have participated fully in the conception and execution of the experimental work, interpretation of the results and manuscript drafting.

Este trabalho foi financiado pela bolsa SFRH / BD / 48497 / 2008 da Fundação para a Ciência e a Tecnologia, Portugal.

Os capítulos de resultados apresentados nesta dissertação incluem um artigo científico publicado, e dois outros em preparação para posterior publicação em revistas científicas internacionais indexadas. Uma vez que estes trabalhos foram realizados em colaboração com outros investigadores, e de acordo com o disposto no nº 1 do Artigo 40º do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, publicado no Diário da República – nº 153, II Série de 5 de Julho de 2003, esclareço que participei integralmente na concepção e execução do trabalho experimental, na interpretação dos resultados e na redação dos manuscritos.

ACKNOWLEDGMENTS

To Professor Sérgio Dias, for making me a scientist. For the persistence, for the patience, for the motivation.

To Professor Gabriela Rodrigues, for the support.

To all the members of the Angiogenesis lab, "minions" old and new, especially to Carla Real, Francisco Caiado, Tânia Carvalho, Ana Bastos, Jacinta Serpa and Ana Gomes, for the scientific discussions and technical support, allied with a great friendship (and to Ana Sofia, Ana Costa, Ana Magalhães, Inês Matias, Germana Domingues, Inês Martins…). Also to the "CIPMers", especially Lara Neto, for the help and joy. The great environment in the lab gave every day a smiling breath!

To the IGC, for the wonderful scientific environment. To the IGC's histopathology, cell imaging and animal facilities stuff.

To Vitor Paixão, for the design.

To my dearest friends (there will always be Colmeosa), for their never-ending support and understanding, for the endless laughs and the plenitude of all moments!

To António Carrilho, for showing me that anything has an endless potential if you keep pushing the limits.

To my *Mãe de Deus* Tonita, for the struggling spirit.

To grandmother Lena, for the happiness of life. To grandmother Ana, for her determination. To grandfather Manuel, for his originality, and for the Paradise. To aunt Eta and uncle Teófilo, for the Summer laughters.

To all my parents for their unconditional love and support. To mother Susana, for the freedom and determination. To father Zé Miguel, for the wonderful example of life, and for the feedback. To Nuno, for the

joy and rearing.

To Rui, for the exquisite tastes of life.

To sister Maria, for making growing up such a wonderful journey! To Miguel, for the authenticity. To Isabel, for cheering us with her life!

To Eduardo, for the love and passion in everything, for the fulfilling, happy life!

RESUMO

A hematopoiese é o processo de formação de células do sangue, que nos mamíferos adultos, em homeostase, ocorre na medula óssea. As especificações da hematopoiese são fornecidas tanto por sinais provenientes das próprias células hematopoiéticas quanto por sinais do microambiente em que se inserem. O microambiente medular inclui, entre outros tipos celulares, células endoteliais que formam o lúmen dos vasos sanguíneos. As células endoteliais da medula óssea regulam a adesão, diferenciação e mobilização de células hematopoiéticas, estando ainda envolvidas na manutenção das células estaminais hematopoiéticas. O estudo da interação entre células endoteliais e células hematopoiéticas permite uma percepção da hematopoiese de maior precisão, tendo por isso um enorme potencial para a descoberta de novas terapêuticas em diversas patologias.

Com esta Tese pretendemos explorar as consequências hematopoiéticas da modulação do microambiente vascular da medula óssea. Para tal, abordámos duas estratégias, que foram previamente relacionadas com um aumento da cobertura vascular em diversos processos fisiológicos e patológicos. A primeira estratégia consistiu em bloquear o Delta-like 4 (Dll4), um ligando da via de sinalização Notch que tem um papel crítico no processo de angiogénese. O bloqueio da sinalização Dll4:Notch em diversos modelos tumorais provoca um aumento da cobertura vascular no tumor, com formação de vasos disfuncionais, que origina uma redução significativa do crescimento tumoral. Estas observações conduziram ao desenvolvimento de terapêuticas associadas à administração de anticorpos neutralizantes anti-Dll4, que se encontram atualmente em ensaios clínicos para tumores sólidos. Os doentes-alvo destes ensaios estão em mielosupressão, e o aumento da cobertura vascular medular está associado a uma recuperação hematopoiética mais rápida. Adicionalmente, foi previamente demonstrado que a sinalização Dll4:Notch está envolvida na hematopoiese, no entanto, em 2008 os efeitos *in vivo* do bloqueio de Dll4 não eram ainda conhecidos. A segunda estratégia

consistiu em modificar o macroambiente através da redução da pressão parcial de oxigénio ambiental (hipóxia). Os mamíferos respondem à redução de oxigénio ambiental essencialmente através de dois mecanismos que garantem a adequada oxigenação dos tecidos: estimulação da eritropoiese e angiogénese. Os efeitos da hipóxia na eritropoiese e angiogénese devem-se essencialmente à modulação da produção de eritropoietina e VEGF, respetivamente. No entanto, a modulação de outras linhagens hematopoiéticas pela hipóxia crónica intermitente não está ainda clarificada, e o papel da interação entre as células endoteliais da medula óssea e células hematopoiéticas nessa modulação é desconhecido.

Os nossos dados da modulação da hematopoiese resultantes do bloqueio de Dll4 demonstraram que este interferia com a sinalização proveniente das próprias células hematopoiéticas. Esta sinalização intrínseca às células hematopoiéticas levou-nos a questionar se células leucémicas expressavam Dll4, e qual o papel da sinalização Dll4:Notch na sobrevivência e proliferação destas células in vitro. De facto, nós e outros tínhamos mostrado que o bloqueio de Dll4 in vivo reduz o tamanho tumoral em modelos murinos de leucemia xenógenos. Adicionalmente, estudos recentes relacionam a expressão de Dll4 com prognóstico em doentes de leucemia.

Nesta Tese investigámos o papel da sinalização Dll4:Notch no compartimento vascular da medula óssea e na hematopoiese. Começámos por observar um modelo terapêutico, em que administrámos um anticorpo neutralizante anti-Dll4 em ratinhos irradiados sub-letalmente (em mielossupressão). Demonstrámos que o bloqueio de Dll4 altera a identidade vascular da medula óssea, aumentando o número de vasos VE-Caderina$^+$, CD31$^+$ e c-kit$^+$, sem modificação do número total de vasos (CD105$^+$), de sinusoides (VEGFR3$^+$), de vasos revestidos por pericitos (SMA$^+$), nem vasos perfundidos (lectina$^+$). Verificámos ainda um aumento de megacariócitos, que estão tipicamente associados à vasculatura, e por isso são considerados parte do compartimento vascular. De

facto, a variação do número de megacariócitos tinha sido previamente relacionada com a quantidade de vasos VE-Caderina$^+$.

Investigámos ainda a modulação da expressão de genes que codificam factores "angiocrine", factores produzidos por células endoteliais que regulam a hematopoiese. O bloqueio de Dll4 induziu a expressão de IGFbp2, IGFbp3, Angpt2, Dll4, DHH e VEGF-A, e reduziu a expressão de FGF1 e CSF2. A expressão dos factores "angiocrine" é dependente da ativação das células endoteliais, e a reconstituição hematopoiética depende da regulação das vias de sinalização Akt e MAPK. Pesquisámos o papel do bloqueio de Dll4 na activação de Akt e MAPK em células endoteliais *in vitro*, e demonstrámos que o bloqueio de Dll4 reduz a fosforilação de Akt e mantém os níveis de fosforilação de MAPK. Para além dos seus efeitos no compartimento vascular, o bloqueio de Dll4 perturbou a hematopoiese, evidenciado pela diminuição de células linfóides B (B220$^+$) e T (CD3$^+$) na medula óssea, e pelo aumento de células mielóides (CD11b$^+$) na medula óssea e sangue periférico. A redução da linfopoiese pode estar relacionada com o aumento de VEGF-A. Ensaios de diferenciação em metilcelulose *in vitro* revelaram que o tratamento anti-Dll4 perturba a comunicação entre células hematopoiéticas e promove a mielopoiese, com aumento de colónias CFU-M e -G. Adicionalmente, a expressão de IGFbp2, IGFbp3 e DHH está relacionada com um aumento de mielopoiese. Analisámos ainda a recuperação hematopoiética após mielossupressão no contexto de transplante de medula. O tratamento anti-Dll4 dos dadores de medula melhora a recuperação linfóide e eritróide de receptores letalmente irradiados. Estes efeitos podem ser explicados pelo aumento de vasos CD31$^+$ e VE-Caderina$^+$ e/ou pelo aumento da expressão de Dll4, IGFbp2 e IGFbp3.

Por fim, observámos o papel do bloqueio de Dll4 especificamente em células endoteliais VE-Caderina$^+$, em ratinhos com uma mutação knockout condicional indutível para Dll4, VECad^{CreERT2}Dll4$^{lox/lox}$. De modo semelhante ao tratamento sistémico, o bloqueio de Dll4 neste modelo

interferiu com o compartimento vascular da medula óssea, com um aumento de vasos VE-Caderina$^+$ e CD31$^+$ e sem modificação do número total de vasos (CD105$^+$), e ainda um aumento de megacariócitos. Em conclusão, o bloqueio de Dll4 interfere com a vasculatura da medula óssea e modela a hematopoiese, e pode ser benéfico durante a recuperação hematopoiética no cenário de transplante de medula.

A interferência do bloqueio de Dll4 com a hematopoiese de forma intrínseca às células hematopoiéticas levou-nos a questionar se diferentes linhas leucémicas expressam Dll4. Nesta Tese mostramos, por Western blotting, que as linhas leucémicas/linfoma HL-60 (leucemia promielocítica aguda, AML), 697 (leucemia linfoblástica aguda pré-B, B-ALL), MOLT-4 (leucemia linfoblástica aguda de células T, T-ALL), HEL (eritroleucemia), JVM-13 (leucemia prolinfocítica B), DoHH2 (linfoma B não-Hodgkin's), Raji (linfoma de Burkitt's), e RCH-ACV (B-ALL) expressam Dll4. Explorámos o papel da sinalização Dll4:Notch na sobrevivência e proliferação de três linhas leucémicas, HL-60, 697 e MOLT-4, representativas de três tipos de leucemia. Para tal, utilizámos um anticorpo neutralizante anti-Dll4, que bloqueia a sinalização Dll4:Notch, uma proteína recombinante humana Dll4, que promove a sinalização Dll4:Notch, e um inibidor de γ-secretase, que inibe a sinalização Notch. Mostramos que nem o bloqueio nem a estimulação da sinalização Dll4:Notch altera a sobrevivência ou proliferação das células leucémicas após 24 horas ou 48 horas de tratamento. A inibição de Notch não altera a sobrevivência das células leucémicas, no entanto, 48 horas de tratamento com o inibidor de γ-secretase promove a paragem do ciclo celular das células leucémicas. Em conclusão, diversas linhas leucémicas e de linfoma expressam Dll4, mas a sinalização Dll4:Notch não afecta diretamente a sobrevivência ou proliferação de células leucémicas in vitro; não obstante, a inibição da sinalização Notch reduz a proliferação de células leucémicas, podendo por isso ser um interessante alvo terapêutico.

Nesta Tese os efeitos da hipóxia crónica intermitente foram avaliadas num modelo animal de apneia obstrutiva do sono. De modo a explorar os efeitos da hipóxia na interação entre células endoteliais e células hematopoiéticas na medula óssea com o mínimo de interferência direta da hipóxia, introduzimos os animais num ambiente de normóxia, subsequente ao período de hipóxia, durante 3 dias. Os nossos resultados preliminares (2 ratos por condição) sugerem que a hipóxia celular na medula óssea é equivalente entre os animais tratados e os controlos após este período. Os nossos resultados preliminares sugerem que a hipóxia aumenta a cobertura vascular na medula óssea (vasos $CD105^+$) e altera a identidade vascular da medula óssea, diminuindo o número de vasos VE-$Caderina^+$ e SMA^+. A frequência de vasos vWF^+ mantém-se inalterada. Os resultados preliminares aqui expostos sugerem ainda uma diminuição do número de megacariócitos e um aumento na apoptose dos megacariócitos na medula óssea. Mais uma vez, a variação do número de megacariócitos parece estar relacionada com a quantidade de vasos VE-$Caderina^+$. Explorámos ainda a expressão de genes "angiocrine" na medula óssea. Os nossos resultados preliminares sugerem um aumento na expressão de IL6, Dll1, CSF2, CSF3, Smad3 e THPO e uma diminuição de IGF1, IGFbp3, IGFbp5, Angpt1, SCF e N-Caderina. Os nossos resultados preliminares sugerem ainda um aumento do número de eritrócitos, monócitos e plaquetas no sangue periférico dos animais sujeitos a um período de hipóxia. Os três parâmetros hematopoiéticos podem ser explicados pelo possível aumento de expressão de IL6. No entanto, o aumento de eritrócitos deve-se provavelmente à estimulação da eritropoiese através ao aumento de eritropoietina. Adicionalmente, o aumento de monócitos pode ser explicado pelo aumento da expressão de CSF2 e CSF3, e o aumento de plaquetas pelo aumento de THPO. Em conclusão, estes dados preliminares sugerem que a hipóxia crónica intermitente, seguida de um período de normóxia, pode modular o microambiente medular, e interferir com a hematopoiese possivelmente através da regulação de genes "angiocrine".

O trabalho realizado neste projeto de doutoramento revelou os efeitos do bloqueio de Dll4 e apresentou resultados preliminares relativos aos efeitos da hipóxia crónica intermitente no microambiente da medula óssea e hematopoiese. Este trabalho permitiu ainda a identificação de domínios microambientais criados por diferentes tipos de vasos na medula óssea. A exploração destes domínios permite uma melhor compreensão dos sinais microambientais que guiam a hematopoiese em homeostase e na doença, o que pode originar o desenvolvimento de terapias altamente específicas e eficientes.

Palavras-chave

Medula óssea; Nicho vascular; Vaso sanguíneo; Hematopoiese; Transplante de medula óssea; Delta-like 4; sinalização Notch; Leucemia; Hipóxia.

SUMMARY

Both cell-intrinsic and environmental cues drive hematopoiesis, which in adult mammals occurs mainly in the bone marrow (BM). BM endothelial cells that line the blood vessels contribute towards the regulation of hematopoiesis. In this Thesis, we used two strategies previously shown to increase vessel numbers, to assess the modulation of the BM "vascular niche" and its effects in hematopoiesis.

The first strategy involved targeting Delta-like 4 (Dll4), a ligand of the Notch pathway family, using neutralizing monoclonal antibodies. We show that Dll4 blockade in vivo modulates BM vessel identity, without changing the overall vessel "quantity", reduces BM lymphocytes and promotes megakaryopoiesis. This modulation of the BM "vascular niche" was accompanied by a modulation of Akt activation in endothelial cells and "angiocrine" genes expression. These modifications resulted in an improvement of hematopoietic recovery following BM transplant. Finally, Dll4 blockade exerts cell-specific effects in the myeloid lineage, promoting myelopoiesis both *in vivo* and *in vitro*.

The modulation of hematopoiesis driven by Dll4 blockade led us to explore its effects on leukemia cells *in vitro*. We show that Dll4 does not modulate leukemia cell survival or growth, and Notch signaling inhibition promotes leukemia cell growth arrest.

As a second strategy, we exposed animals to chronic intermittent hypoxia (CIH), followed by a period of normoxia. Our preliminary data suggest CIH modulates the BM vessel identity and increases the overall vessel "quantity". This modulation of the BM "vascular niche" was accompanied by modulation of "angiocrine" genes expression. Our preliminary results show that CIH modulates megakaryopoiesis and increases circulating erythrocytes and monocytes.

Taken together, our results suggest tampering with the BM vascular niche, by Dll4:Notch signaling blockade or by exposure to CIH, affects

hematopoiesis by favoring specific hematopoietic lineages. These data may be relevant in a setting of BM transplantation or BM diseases onset and progression.

Keywords

Bone marrow; Vascular niche; Blood vessel; Hematopoiesis; Bone marrow transplant; Delta-like 4; Notch signaling; Leukemia; Hypoxia

ABBREVIATIONS LIST

ADAM	A disintegrin and metalloproteases
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
Angpt	Angiopoietin
B	B lymphocyte
Ba	Basophil
BC	Before Christ
BFU-E	Burst forming unit-erythrocyte
BM	Bone marrow
BMP	Bone morphogenetic protein
BMT	Bone marrow transplant
β2MG	β2-microglobulin
CBC	Complete blood count
CD	Cluster of differentiation
CFU	Colony forming unit
CFU-E	Colony forming unit-erythrocyte
CFU-G	Colony forming unit-granulocyte
CFU-GM	Colony forming unit-granulocyte-macrophage
CFU-GEMM	Colony forming unit-granulocyte-erythrocyte-macrophage-megakaryocyte
CFU-M	Colony forming unit-monocyte
CFU-Mk	Colony forming unit-megakaryocyte
CIH	Chronic intermittent hypoxia
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CSF	Colony stimulating factor
CXCR4	C-X-C chemokine receptor type 4
D	Dendritic cell
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DCP	Dendritic common progenitor
DHH	Desert hedgehog
Dll	Delta-like
G-CSF	Granulocyte colony-stimulating factor
GMP	Granulocyte and macrophage progenitor

E	Embryonic day
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
Eo	Eosinophil
EPC	Endothelial progenitor cell
Er	Erythrocyte
E-selectin	Endothelial selectin
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Flk-1	Fetal liver kinase-1
Hh	Hedgehog
HIF	Hypoxia inducible factor
HPC	Hematopoietic progenitor cell
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HSPC	Hematopoietic stem/progenitor cell
HSC	Hematopoietic stem cell
HUVEC	Human umbilical cord vein endothelial cell
IGF	Insulin-like growth factor
IGFbp	Insulin-like growth factor binding protein
IL	Interleukin
IP	Intraperitoneally
Lin	Lineage
LT-HSC	Long-term hematopoietic stem cell
M	Monocyte
Maml	Mastermind-like [transcription coactivator]
MAPK	Mitogen-activated protein kinase
MCAM	Melanona-associated cell adhesion molecule
M-CSF	Macrophage colony-stimulating factor
MEP	Megakaryocyte and erythrocyte progenitor
Mϕ	Macrophage
Mk	Megakaryocyte
MMP	Matrix metalloprotease
MPC	Mesenchymal progenitor cell
MPP	Multipotent progenitor [cell]
MSC	Mesenchymal stem cell
N	Neutrophil
NECD	Notch extracellular domain

NICD	Notch intracellular domain
NK	Natural killer [cell]
P	Platelet
PB	Peripheral blood
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PHD	Prolyl hydroxylase
pO2	Oxygen tension
Pre-pro-B	Early B lymphocyte progenitor
Pre-B	Late B lymphocyte progenitor
Pro-B	B lymphocyte progenitor
Pro-NK	Natural killer progenitor
Prx1	Paired related homeobox protein 1
PSGL1	P-selectin glycoprotein 1
PTH	Parathyroid hormone
rhDll4	Recombinant human Delta-like 4
ROS	Reactive oxygen species
SCF	Stem cell factor
Sca-1	Stem cell antigen-1
SDF-1	Stromal cell-derived factor-1
SEC	Sinusoidal endothelial cell
SMA	Smooth muscle actin
ST-HSC	Short-term hematopoietic stem cell
T	T lymphocyte
Tie-2	Tyrosine kinase with immunoglobulin-like EGF-like domains 2
TGF-β	Transforming growth factor-β
THPO	Thrombopoietin
Treg	T regulatory lymphocytes
Ty	Thymocyte
VCAM-1	Vascular cell adhesion protein 1
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	von Hippel-Lindau protein
vWF	von Willebrand factor

CONTENTS

Acknowledgments iii
Resumo v
Summary xi
Abbreviations List xiii
Contents xvi
Figures Index xx
Tables Index xxii

CHAPTER 1 - INTRODUCTION 1

HEMATOPOIESIS 3
MACROENVIRONMENTAL CUES THAT PERTURB HEMATOPOIESIS 4
MICROENVIRONMENTAL CUES THAT PERTURB HEMATOPOIESIS 6
Osteoblasts 8
Osteoclasts 12
Adipocytes 13
Regulatory T lymphocytes 14
Macrophages and monocytes 14
Neurons also modulate the BM microenvironment and hematopoiesis 15
Nonmyelinating Schwann cells 15
Mesenchymal stem/progenitor cells 17
Osterix$^+$ MPCs 17
Prx1$^+$ MPCs 18
MCAM$^+$/Nestin MSCs 19
Leptin receptor$^+$ MSCs 20
Endothelial cells 20
MAJOR SIGNALING PATHWAYS DRIVING HEMATOPOIESIS 25
THE NOTCH SIGNALING PATHWAY 26
An overview 26
Dll4 and angiogenesis 28

Dll4 and hematopoiesis..... 31
Malignant hematopoiesis: Leukemia..... 33
Notch signaling in Leukemia 34
AIMS..... 35
REFERENCES..... 35

CHAPTER 2 - CONTEXT- AND CELL-DEPENDENT EFFECTS OF DELTA-LIKE 4 TARGETING IN THE BONE MARROW MICROENVIRONMENT 49

ABSTRACT..... 50
INTRODUCTION 51
METHODS..... 53
Animals and experimental design..... 53
Sample collection..... 54
Bone Marrow Transplants 54
Cell culture..... 54
In vitro colony forming assays..... 55
Flow cytometry..... 55
Histological and immunohistochemical analysis 57
Vascular perfusion 58
Western blotting..... 58
Reverse transcriptase PCR (RT–PCR)..... 58
Statistical analysis..... 59
RESULTS 62
Systemic anti-Dll4 treatment interferes with the BM vascular niche 62
Specific effects of anti-Dll4 treatment on endothelial cells 67
Anti-Dll4 treatment perturbs hematopoietic recovery following irradiation..... 71
Anti-Dll4 treatment of donor BM improves hematopoietic recovery following transplantation into lethally irradiated recipients..... 74
DISCUSSION 75

REFERENCES **78**

CHAPTER 3 - LEUKEMIA CELL CYCLE ARREST INDUCED BY DELTA-LIKE 4 (DLL4)-INDEPENDENT NOTCH SIGNALING INHIBITION **83**

ABSTRACT **84**

INTRODUCTION **85**

METHODS **87**

Cell culture 87

Western blotting 87

Apoptosis assay 88

Cell cycle analysis 88

Statistical analysis 89

RESULTS **90**

Several leukemia and lymphoma cell lines express Dll4 90

Notch signaling pathway does not affect spontaneous leukemia cell apoptosis 91

Notch signaling pathway promotes leukemia cell growth arrest in a Dll4 independent manner 94

DISCUSSION **97**

REFERENCES **98**

CHAPTER 4 - CHRONIC INTERMITTENT HYPOXIA AFFECTS HEMATOPOIESIS AND MODULATES THE BONE MARROW MICROENVIRONMENT **103**

ABSTRACT **104**

INTRODUCTION **105**

METHODS **107**

Animals and experimental design 107

Sample collection 108

Pimonidazole Staining 108

Histological and immunohistochemical analysis 108

Reverse transcriptase PCR (RT–PCR) 109

RESULTS **110**
Chronic intermittent hypoxia modulates hematopoiesis 110
Chronic intermittent hypoxia interferes with the BM vascular niche 112
Chronic intermittent hypoxia modulates bone marrow "angiocrine" gene expression 116
DISCUSSION **118**
REFERENCES **121**

CHAPTER 5 - DISCUSSION **129**

THE CONSEQUENCES OF SYSTEMIC DLL4 BLOCKADE IN THE BM MICROENVIRONMENT AND HEMATOPOIESIS **131**

THE ROLE OF CHRONIC INTERMITTENT HYPOXIA, A MODEL FOR OBSTRUCTIVE SLEEP APNEA, IN THE BM MICROENVIRONMENT AND HEMATOPOIESIS **139**

CONCLUDING REMARKS **143**
REFERENCES **145**

APPENDIX - COMMUNICATION BETWEEN BONE MARROW NICHES IN NORMAL BONE MARROW FUNCTION AND DURING HEMOPATHIES PROGRESSION **A1**

FIGURES INDEX

Figure 1.1. Model of the hematopoietic hierarchy. 3
Figure 1.2. Schematic overview of the hypoxia inducible factor (HIF) pathway. 5
Figure 1.3. Model of the bone marrow microenvironment. 6
Figure 1.4. The hematopoietic cues in the niche: the role of osteoblasts, osteoclasts and adipocytes. 9
Figure 1.5. The hematopoietic cues in the niche: the role of Schwann cells, Osterix^{+} mesenchymal progenitor cells (MPCs), Prx1^{+} MPCs, Leptin^{+} mesenchymal stem cells (MSCs) and endothelial cells. 16
Figure 1.6. Schematic overview of the Notch signaling pathway. .. 27
Figure 1.7. Dll4 regulates angiogenesis. 29
Figure 1.8. The role of Dll4 in hematopoiesis. 32
Figure 2.1. Therapeutic anti-Dll4 blockade interferes with the BM vascular niche. 63
Figure 2.2. Anti-Dll4 blockade interferes with the BM vascular niche. 64
Figure 2.3. Different endothelial cell markers reveal different types of BM vessels. 65
Figure 2.4. Endothelial cell-specific Dll4 blockade interferes with the BM vascular niche. 66
Figure 2.5. Therapeutic anti-Dll4 blockade interferes with the hepatic vascular niche. 67
Figure 2.6. Endothelial-specific effects of anti-Dll4 treatment. 68
Figure 2.7. Endothelial-specific effects of anti-Dll4 treatment. 70
Figure 2.8. Anti-Dll4 treatment perturbs hematopoiesis following irradiation. 72
Figure 2.9. Anti-Dll4 treatment perturbs hematopoiesis following irradiation. 73
Figure 2.10. Anti-Dll4 treatment of donor BM improves hematopoietic recovery following transplantation into lethally irradiated recipients. 74

Figure 3.1. Several leukemia and lymphoma cell lines express Dll4 90
Figure 3.2. Modulation of Dll4-mediated Notch signaling and canonical Notch signaling inhibition does not affect leukemia cell apoptosis. 93
Figure 3.3. Notch signaling inhibition reduces leukemia cell proliferation, independently of Dll4. 95
Figure 3.4. Leukemia living cell counts and percentage are not modulated by Dll4 or Notch signaling. 96
Figure 4.1. Experimental design. 108
Figure 4.2. Chronic intermittent hypoxia modulates hematopoiesis. 111
Figure 4.3. Bone marrow cell count. 112
Figure 4.4. Chronic intermittent hypoxia interferes with the BM vascular niche. 114
Figure 4.5. Chronic intermittent hypoxia modulates bone marrow angiocrine gene expression. 117
Figure 5.1. Endothelial-specific Dll4 blockade does not affect the main hematopoietic lineages. 134
Figure 5.2. Anti-Dll4 blockade, through Notch1 inhibition, impairs B lymphopoiesis in a hematopoietic cell-specific manner. 134
Figure 5.3. Endothelial-specific Dll4 blockade in donor BM improves hematopoietic recovery following transplantation into lethally irradiated recipients. 136
Figure 5.4. Recipient BMT mice treated with anti-Dll4 developed signs of severe morbidity by the first week after transplantation. 136
Figure 5.5. Representative figures of the major findings included in Chapter 2. 138
Figure 5.6. Representative figures of the major findings included in Chapter 3. 142
Figure 5.7. Models of the bone marrow microenvironment. 143

TABLES INDEX

Table 1.1. Major macroenvironmental cues that perturb hematopoiesis. 4
Table 1.2. Major macroenvironmental cues that perturb hematopoiesis. 25
Table 1.3. Dll4 is crucial for developmental angiogenesis. 30
Table 2.1. Antibodies list. 56
Table 2.2. Primers list. 60
Table 4.1. Antibodies list. 109
Table 4.2. Primers list. 110

1

INTRODUCTION

CONTENTS

HEMATOPOIESIS 3

MACROENVIRONMENTAL CUES THAT PERTURB HEMATOPOIESIS 4

MICROENVIRONMENTAL CUES THAT PERTURB HEMATOPOIESIS 6

Osteoblasts 8

Osteoclasts 12

Adipocytes 13

Regulatory T lymphocytes 14

Macrophages and monocytes 14

Neurons 15

Nonmyelinating Schwann cells 15

Mesenchymal stem/progenitor cells 17

Osterix^{+} MPCs 17

Prx1^{+} MPCs 18

MCAM^{+}/Nestin MSCs 19

Leptin receptor^{+} MSCs 20

Endothelial cells 20

MAJOR SIGNALING PATHWAYS DRIVING HEMATOPOIESIS 25

THE NOTCH SIGNALING PATHWAY 26

An overview 26

Dll4 and angiogenesis 28

Dll4 and hematopoiesis 31

Malignant hematopoiesis: Leukemia 33

Notch signaling in Leukemia 34

AIMS 35

REFERENCES 35

"Thy bone is marrowless. Thy blood is cold…"

Shakespeare's *The Tragedy of Macbeth*, 17th century

The bone marrow (BM) has always intrigued scientists; already in the 4th century BC the function of this tissue was speculated – Hyppocrates and Galen defended the BM was the bone's nutritional matrix, whereas Aristotle believed it was an "excrement" of the bone [1,2]. In the 17th century, soon after Hooke's first description of the cell [3], scientists were still suggesting new theories for the use of the marrow to the bone [1,4]. It was only after the first descriptions of BM cells [5] that, late in the 19th century, Neumann and Bizzozero revealed hematopoiesis (the generation of blood cells) occurred in the BM [2,6,7]. Later works led to the discovery that hematopoietic stem cells (HSCs) residing in the BM were responsible for supplying all blood cells throughout life [8-14]. This knowledge was taken to clinical practice in the 1950s, with the first BM transplants (BMTs) performed in radio and chemotherapy-treated patients [15], which led to the development of therapies of hematological diseases, solid tumors and immune disorders [15-17]. These findings and clinical applications also generated great interest in exploring the BM physiology. In fact, soon after the discovery that hematopoiesis occurred in the BM, researchers noted that there were different cell types in the BM microenvironment, leading to the general idea of an "organizational matrix" somehow coordinating the localization, expansion and death of the different hematopoietic elements [18]. This led to the concept, in the 20th century, of microenvironmental signals regulating hematopoiesis which, together with its underlying molecular organization(s), have been under intense scrutiny over the past 50 years [19-29].

This Thesis focuses on the BM microenvironment, and how molecular cues perturb it and modulate hematopoiesis.

HEMATOPOIESIS

The process by which a HSC differentiates into mature, functional blood cells, is called hematopoiesis. Hematopoiesis arises in the embryo from a putative common precursor of hematopoietic and endothelial cells (ECs), called the hemangioblast, through a hemogenic endothelium intermediate [30-36]. Hematopoiesis begins in the yolk sac, then the aorta-gonad-mesonephros region, placenta, viteline and umbilical arteries, the fetal liver and spleen, and ultimately in the BM [37-39]. In adult physiological conditions, hematopoiesis is confined primarily to the BM, however, in pathological conditions it may occur in the spleen, liver, and occasionally lung, kidney, heart and brain [40-44]. Daily, the adult human BM is estimated to produce hundreds of billions of hematopoietic cells and platelets, which replace others due to senescence, utilization or recirculation [2,45-48].

Hematopoietic stem cells are the source of all hematopoiesis, and can be divided in two sub-groups, long-term (LT) reconstituting HSCs (LT-HSCs), which have unlimited self-renewal capacity, and short-term (ST-) HSCs, which have a self-renewal capacity of approximately 8 weeks [19,49]. These then

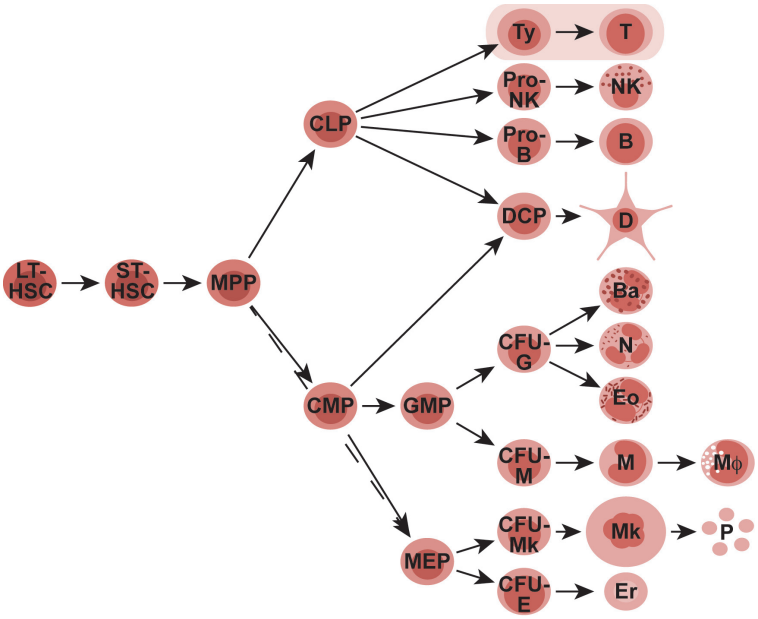


Figure 1.1. Model of the hematopoietic hierarchy.
Hematopoiesis occurs in the BM, with the exception of T lymphocyte differentiation (shaded area), that occurs in the thymus. **LT-HSC**, long-term hematopoietic stem cell. **ST-HSC**, short-term hematopoietic stem cell. **MPP**, multipotent progenitor cell. **CLP**, common lymphoid progenitor. **CMP**, common myeloid progenitor. **GMP**, granulocyte and macrophage progenitor. **MEP**, megakaryocyte and erythrocyte progenitor. **Ty**, thymocyte. **Pro-NK**, natural killer progenitor. **Pro-B**, B lymphocyte progenitor. **DCP**, dendritic common progenitor. **CFU-G**, colony forming unit-granulocyte. **CFU-M**, colony forming unit-monocyte. **CFU-Mk**, colony forming unit-megakaryocyte. **CFU-E**, colony forming unit-erythrocyte. **T**, T lymphocyte. **NK**, natural killer cell. **B**, B lymphocyte. **D**, dendritic cell. **Ba**, basophil. **N**, neutrophil. **Eo**, eosinophil. **M**, monocyte. **Mk**, megakaryocyte. **Er**, erythrocyte. **Mϕ**, macrophage. **P**, platelets.

differentiate into multipotent progenitors (MPPs), which further differentiate into common lymphoid or myeloid progenitors (CLP or CMP, respectively), generally referred to as hematopoietic progenitor cells (HPCs) [50-55]. The lymphoid lineage ultimately gives rise to T and B lymphocytes, and natural killer (NK) cells [52]. The myeloid lineage comprises two additional progenitors, the granulocyte and macrophage progenitor (GMP) and the megakaryocyte and erythrocyte progenitor (MEP); the latter may also arise from MPPs [54,56]. Both CLP and CMP can differentiate to dendritic cells [57] (Figure 1.1).

MACROENVIRONMENTAL CUES THAT PERTURB HEMATOPOIESIS

The hematopoietic system is very dynamic. It responds to exogenous stimuli by regulating the production of cells (proliferation / differentiation), or their mobilization into the peripheral blood (Table 1.1).

Table 1.1. Major macroenvironmental cues that perturb hematopoiesis.

Macroenvironmental cues / stimuli	Hematopoietic effects
Hypoxia	Anemia (reduced blood erythrocytes and hemoglobin), followed by an increase in erythropoiesis [58]
Irradiation	BM aplasia (reduced cell number), anemia and leukopenia (reduced blood leukocytes) followed by HSC proliferation and differentiation [59-61]
Bleeding	Anemia, followed by increased erythropoiesis and HSCs self-renewal and proliferation [62-64]
Cytotoxic agents	Leukopenia and thrombocytopenia (decreased blood platelets), followed by HPC proliferation and mobilization, lymphopoiesis, myelopoiesis and thrombocytosis [65-70]
Infection	HSC proliferation, HPC proliferation or ablation, lymphopoiesis or myelopoiesis or lymphoid or myeloid depletion, binary GMP fate choice, HSC, HPC, myeloid or lymphoid mobilization [71-73]
Dietary protein restriction	BM aplasia, erythrocytosis (increased erythrocyte mass), leukopenia and thrombocytopenia [74]
Dietary cholesterol	Thrombocytosis, neutrophilia, B lymphocytosis and hematopoietic stem/progenitor cell (HSPC) mobilization [75]
Diabetes	Poor HSPC mobilization [76]

In this Thesis we explored the hypoxia effects in hematopoiesis and the BM microenvironment.

It has long been realized that the environment may influence hematopoiesis. The more striking effect is the one induced by environmental hypoxia (low oxygen pressure). In the 19th century it was first reported that in high altitudes, where oxygen pressure is low, erythropoiesis is stimulated [58]. This was later revealed to be driven by erythropoietin [77], a hormone synthesized in the kidney that targets late erythroblast precursors (CFU-E – see figure 1.1), and increases mature erythrocyte levels by preventing apoptosis [58]. Erythropoietin is now being used to treat several kinds of anemia [42].

Besides promoting erythropoiesis, hypoxia also promotes plasmacytoid dendritic cell differentiation, delays megakaryocyte differentiation and is required for the maintenance of self-renewal and quiescence of HSCs [78-89]. However, these studies reveal the role of hypoxia on cultured cells, and *in vivo* studies are based on genetic and pharmacologic targeting of hypoxia-inducible factors (HIFs). We consider these reports may not fully comprise the effects of environmental hypoxia.

The mechanism by which hypoxia modulates hematopoiesis is believed to involve the stabilization of HIF-α [87,88,90]. The HIFs are transcription activators that function as master regulators in oxygen

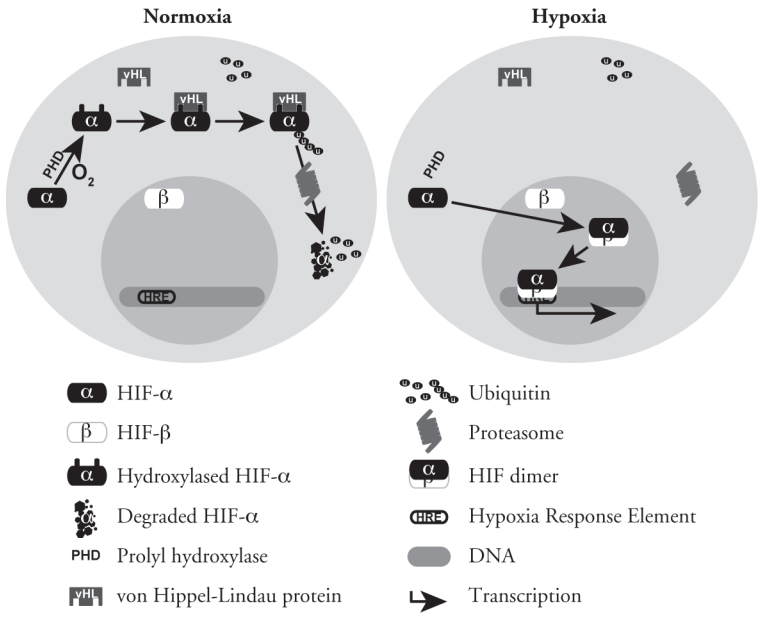


Figure 1.2. Schematic overview of the hypoxia inducible factor (HIF) pathway.

homeostasis. They are protein heterodimers which consist of an alpha subunit, directly regulated by oxygen levels, and a beta subunit, constitutive nuclear proteins which also participate in other transcriptional responses [91]. In well-oxygenated conditions, a specific proline residue in HIF-α is hydroxylased by the prolyl hydroxylases (PHD)1, 2 or 3, which use O_2 as a substrate [92,93]. The von-Hippel Lindau protein (VHL) then binds to the hydroxylased HIF-α, and recruits an ubiquitin ligase that targets HIF-α for proteasomal degradation [94]. In hypoxic conditions, HIF-α subunit is not degraded, and forms a dimer with the constitutive HIF-β; this heterodimer binds to hypoxia regulated elements, present in promoters and enhancers of several genes, promoting its transcription [95] (Figure 1.2).

MICROENVIRONMENTAL CUES THAT PERTURB HEMATOPOIESIS

Besides hematopoietic cells, the BM is constituted of stromal cells: osteoblasts, osteoclasts, adipocytes, neurons, Schwann cells, Osterix$^+$ mesenchymal progenitor cells (MPCs), paired related homeobox protein 1 (Prx1) MPCs, Nestin$^+$/melanoma-associated cell adhesion molecule (MCAM)$^+$ mesenchymal stem cells (MSCs), Leptin receptor$^+$ MSCs and ECs (Figure 1.3). One of the first demonstrations of the involve-

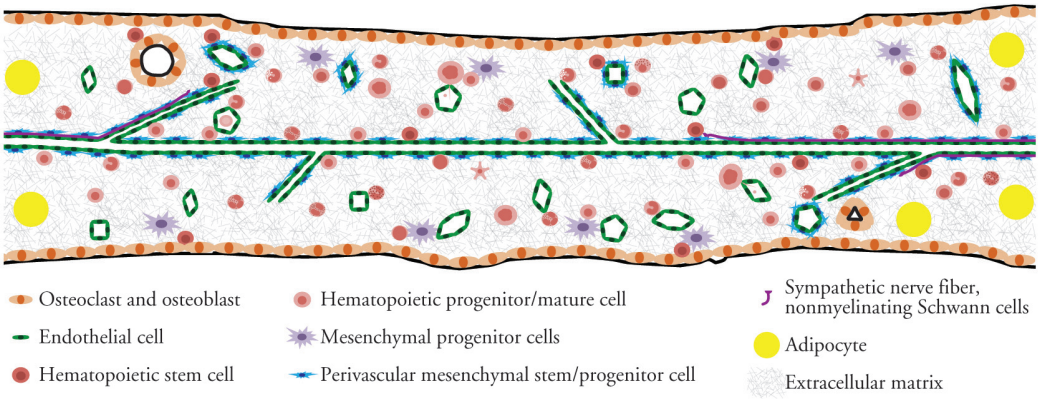


Figure 1.3. Model of the bone marrow microenvironment.

ment of stromal cells in hematopoiesis was provided in 1977, when long-term hematopoietic cell cultures were established by co-culturing hematopoietic cells with stromal cells [96]. This led to a theory proposed by Schofield the following year that stated the existence of two distinct BM niches with defined functions that would support hematopoiesis in a stratified manner – the endosteal niche, where osteoblasts represent the major cell type and where HSCs are believed to remain quiescent, and the vascular niche, where ECs represent the major cell type and HSCs are believed to proliferate and undergo differentiation and ultimately are (as differentiated progeny) mobilized to the circulation [22]. This theory has prevailed until now – in fact, several evidences challenging it were published recently, with the most striking data contradicting the role of the "endosteal" and "vascular" niches in HSCs biology being published since 2008 [29,86,97-99]. In fact, the recent discoveries on the BM microenvironment have been remarkable – four of the ten enumerated stromal cell types have emerged as hematopoiesis-supportive during the past four years [28,98-101].

Both osteoblasts and ECs expand HSC numbers *in vitro*, and its co-transplantation with HSCs increases the engraftment rate [102-105], such that it seems that similarly to stromal cells, both EC and osteoblasts support HSCs expansion and eventually differentiation *in vitro* and *in vivo*, independently of HSC localization to a particular niche, as proposed by Schofield. Furthermore, the original idea whereby modulating osteoblast numbers the overall BM HSC count would linearly vary has been intensively challenged [97,106-109]. The idea that HSCs must reside in non-perivascular areas, because of its hypoxic state and poor perfusion, can be a misinterpretation: the blood flow rate in BM sinusoids can be as low as 0-0.2mm/sec, 10-20 times lower than in arteries, explaining both the poor perfusion of HSCs and its hypoxic state [27,87,110,111]. Likewise, HSCs were found to localize in the sinusoidal hypoxic niche [86]. The identification of novel HSC markers have further allowed concise anatomical studies, and in fact, even though HSCs were found

predominantly in the metaphysis (the extremities of long bones, high trabecular (bone)-rich areas), HSCs are more adjacent to the blood vessels than to the bone and most osteoblasts are adjacent to the vasculature [19,21-24,26,29,112-114]. In 2013, this was clarified by the identification of the endosteal niche as a lymphopoiesis regulator, supporting lymphoid HPCs, and the perivascular niche-supporting HSCs function [98,99]. Different BM microenvironments, created by different types and activation states of cells and cell-cell interactions, most likely drive different stages of hematopoiesis.

In this Thesis, I will present the BM microenvironment as a reflection of each particular cell type and its molecular cues that were shown to drive hematopoiesis. Besides the stromal cells, BM mature hematopoietic cells also modulate BM hematopoiesis and are included in this section.

Osteoblasts

Osteoblasts are the major bone-forming cells, and were the first to arise as important microenvironmental cells that regulate HSCs biology. The first direct evidences were published in 2003, in two seminal papers of the same Nature issue, suggesting a stem cell-supportive osteoblastic niche *in vivo*, whereby modulation of osteoblastic numbers in the BM correlated with HSCs numbers [106,108]. However, as will be discussed later in this section, this correlation was strongly challenged over the last few years.

Osteoblasts regulate HSCs chemotaxis (migration towards a specific localization) and adhesion, retaining HSCs in the BM, which, according to Schofield's theory, would be characteristic of both niches. It also regulates survival, quiescence and self-renewal capacity (accessed by HSCs reconstitution potential), which is characteristic of the endosteal niche. Interestingly, it also regulates hematopoietic stem/progenitor cell (HSPC) differentiation, which would be characteristic of the vascular niche, according to Schofield [22] (Figure 1.4).

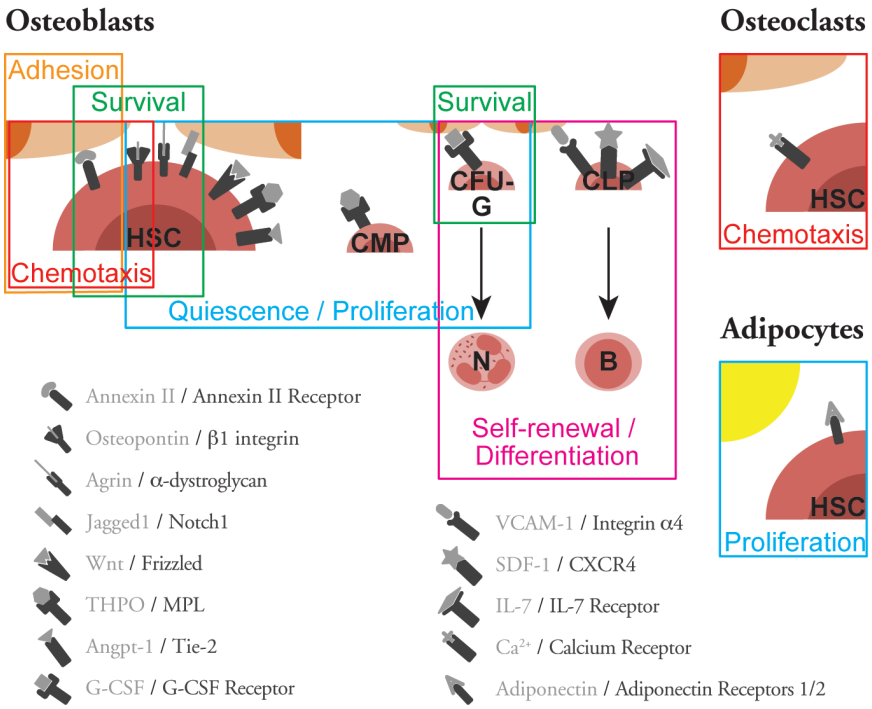


Figure 1.4. The hematopoietic cues in the niche: the role of osteoblasts, osteoclasts and adipocytes. **HSC**, hematopoietic stem cell. **CMP**, common myeloid progenitor. **CFU-G**, colony forming untit-granulocyte. **CLP**, common lymphocyte progenitor. **N**, neutrophil. **B**, B lymphocyte.

Osteoblasts regulate HSCs chemotaxis, retaining HSCs in the BM

Endothelial cells and osteoblasts express annexin II, which through annexin II receptor promote HSCs engraftment. In a BM transplantation setting in which the recipient mice were treated with annexin II peptide, thereby blocking endogenous annexin II function recipients exhibited a reduced BM transplanted cell percentage and chimerism [115].

Osteopontin, a matrix glycoprotein produced by osteoblasts, localize HSCs to the endosteal surface via β_1 integrin subunit; osteopontin$^{-/-}$ mice exhibit aberrant HSPCs localization when subjected to BMT [116] (Figure 1.4).

Osteoblasts promote HSCs adhesion

Annexin II receptor promotes HSCs adhesion, as assessed by adhesion assays *in vitro* in which annexin II blockade reduced adherent HSCs, and annexin II overexpression in the stromal cell layer increased adherent HSCs [115] (Figure 1.4).

Osteoblasts regulate HSCs survival

Annexin II receptor promotes HSCs survival. Annexin II$^{-/-}$ mice exhibit reduced BM HSCs numbers, and in transplantation assays in which the recipient mice were treated with annexin II peptide, thereby blocking endogenous annexin II function, there was a reduced survival and chimerism [115].

Osteopontin is a negative regulator of hematopoiesis, decreasing HSCs survival; osteopontin$^{-/-}$ mice exhibit reduced HSCs apoptosis and increased BM HSCs number and frequency [116,117].

Agrin, an extracellular matrix proteoglycan, is expressed by osteoblasts and MSCs and promotes a-dystroglycan receptor-expressing HSC survival; agrin$^{-/-}$ mice exhibit increased apoptotic HSCs and reduced hematopoietic cells in the BM, spleen and peripheral blood (PB) [118].

Jagged1 expressing osteoblasts support HSC survival, as HSCs cultured with Jagged1 *in vitro* exhibit enhanced survival *in vivo* [106,119] (Figure 1.4).

Osteoblasts regulate HSCs quiescence/proliferation

Osteopontin is a negative regulator of hematopoiesis, promoting HSCs quiescence; osteopontin$^{-/-}$ mice exhibit more cycling HSCs and increased BM HSCs number and frequency [116,117].

Agrin promotes HSC proliferation; agrin$^{-/-}$ mice exhibit reduced hematopoietic cell proliferation and decreased hematopoietic cells in the BM, spleen and PB [118].

Jagged1 expressing osteoblasts support HSC proliferation, assessed in mouse models in which increased Jagged1 osteoblast expression increases BM HSC numbers, and HSCs cultured with Jagged1 *in vitro* exhibit enhanced expansion *in vivo* [106,119].

Wnt ligands of both the noncanonical and canonical pathways are expressed by osteoblasts. The shift between noncanonical and canonical Wnt signaling activation in HSCs determinates HSCs quiescence or proliferation, respectively [120].

Thrombopoietin (THPO)-expressing osteoblasts localize HSCs to the endosteum and promote HSC quiescence and HPC proliferation; $Thpo^{-/-}$ mice exhibit increased HSC proliferation and reduced HSC numbers [121-124].

Angiopoietin (Angpt)-1, expressed by osteoblasts (and $Nestin^{+}$ MSCs), activates tyrosine kinase with immunoglobulin-like EGF-like domains 2 (Tie-2) in HSCs, promoting its quiescence, as $Tie\text{-}2^{+}$ phenotypic HSCs are long-label retaining cells at the G_0 phase of the cell cycle [112,125]. Additionally, Angpt-1-mediated Tie-2 signaling in HSCs indirectly promotes its adhesion, by increasing β_1 integrin subunit expression in HSCs [112] (Figure 1.4).

Osteoblasts regulate HSPCs differentiation

Osteoblasts support myelopoiesis by expressing membrane-bound granulocyte colony-stimulating factor (G-CSF), which increases HPCs survival, proliferation and differentiation [126].

Osteoblasts support B-lymphopoiesis by expressing vascular cell adhesion protein-1 (VCAM-1), SDF-1 and interleukin-7 (IL-7), which activate integrin $\alpha 4$ subunit, C-X-C chemokine receptor type 4 (CXCR4) and IL-7 receptor in CLPs, required for CLP adhesion and differentiation to pre-pro-B (early B lymphocyte progenitor) and pro-B differentiation stages, respectively [99,127]. Abrogation of stromal cell-derived

factor-1 (SDF-1) in osteoblasts (in Col2.3CreSDF-1$^{fl/fl}$ mice) reduces the number of early lymphoid precursors (lymphoid-primed MPPs and CLPs), and reconstitution assays reveal a failure of reconstitution of the lymphoid, but not myeloid, lineages [99] (Figure 1.4).

It is noteworthy that previously identified osteoblastic cues, still cited in original research papers and reviews, were recently recognized as being a result of data misinterpretation. Zhang et al. suggested that the adhesion of HSCs to osteoblasts would be mediated by N-Cadherin; however, detailed HSC characterization has later proved that HSCs do not express N-Cadherin, thus, this is not a relevant microenvironmental cue [108,128-131]. Furthermore, Calvi et al. described that increased osteoblastic numbers, in a murine model of parathyroid hormone (PTH) receptor activation specifically in osteoblasts, corresponded to increased BM HSC number and engraftment; this finding was challenged in 2012 by Calvi herself, who showed that PTH receptor activation specifically in osteocytes (terminally differentiated osteoblasts) increases osteoblasts, osteoclasts and trabecular bone, but is not sufficient to induce phenotypic or functional changes in BM HSCs [97,106]. Interestingly, in her original paper, Calvi described that the transgenic mice targeting PTH receptor in the osteoblastic lineage did not increase solely osteoblasts, but also osteoblast precursors (MPCs) which, as will be later mentioned, support and maintain HSCs in the niche [28,125,132].

Osteoclasts

Osteoclasts are specialized bone-resorbing cells that secrete several proteolytic enzymes and release factors from the bone degradation, including calcium. Osteoclasts retain HSCs in the BM.

Osteoclasts regulate HSCs chemotaxis, retaining HSCs in the BM

A calcium-sensing receptor, expressed mainly by HSCs in the BM, localize HSCs to calcium-rich endosteal areas; calcium-sensing receptor$^{-/-}$

mice exhibit lower BM mononuclear cell and HSC count, extramedullary hematopoiesis and decreased adhesion to the extracellular matrix components fibronectin and collagen I [133] (Figure 1.4).

The role of osteoclasts in hematopoiesis is not well elucidated; besides the above-mentioned study, in which the osteoclast role is indirect, some other studies revealed inconsistent results. Osteoclast-secreted bone-resorbing proteinases, including matrix metalloprotease (MMP)-9, were proposed to cleave SDF-1 and stem cell factor (SCF) and reduce osteopontin, mobilizing HSPCs to the PB in a CXCR4-dependent manner; inhibition of osteoclasts with calcitonin (which additionally lowers calcium PB level) reduces HSPC mobilization in homeostasis, bleeding and infection [134-136]. However, using three osteopetrosis mouse models, op/op (loss-of-function mutation in macrophage-CSF, M-CSF), c-Fos$^{-/-}$ and receptor activator of nuclear factor kappa B ligand, RANKL$^{-/-}$, and a bisphosphonate-based therapeutic model, in which osteoclasts are absent or inhibited, G-CSF induced HSC mobilization yielded different results [137]. Furthermore, mouse models for both decrease and increase of osteoclasts revealed reduced or unaltered HSPCs BM content. Inhibition of osteoclast function by bisphosphonate treatment reduces BM HSCs numbers and engraftment, promotes its proliferation and differentiation, and abolishes PTH-mediated HSC expansion [106,138]. On the other hand, a model for increased osteoclastogenesis, CREB-binding protein$^{+/-}$ mice, exhibit decreased BM LT-HSCs [139]. In addition, as previously mentioned, PTH receptor activation in osteocytes increases osteoclasts but does not alter BM HSCs [97].

Adipocytes

Adipocytes are the most common stromal cells in the BM, and their number increases with age and vary greatly with the bone type [140]. Adipocytes are negative regulators of hematopoiesis, impairing HSCs proliferation. Comparisons between adipocyte-rich bones, such as tail

vertebrae, and adipocyte-free bones, such as the vertebrae of the thorax, revealed reduced LT- and ST-HSCs frequency in adipocyte-rich bones; furthermore, adipocyte-free mice are capable of accelerated BM engraftment after irradiation [141].

Adipocytes promote HSCs proliferation

Adiponectin, an adipocyte-specific hormone, binds to adiponectin receptors 1 and 2, expressed by HSCs, and increases the proliferation of functionally immature HSCs; *in vitro* culture of HSPCs with adiponectin increases HSPC number and chimerism in competitive transplants [142] (Figure 1.4).

Regulatory T lymphocytes

Regulatory T lymphocytes (T_{reg}) create an immunosuppressive microenvironment where HSPCs reside. This immunosuppressive microenvironment is imperative for allogenic BMT [143].

Macrophages and monocytes

Macrophages and its precursors, monocytes (Figure 1.1), are phagocytes. Macrophages and monocytes retain HSCs in the BM and promote its quiescence.

Macrophages and monocytes regulate HSCs chemotaxis, retaining HSCs in the BM

Bone marrow monocyte and macrophage selective depletion, in a pharmacological model (administration of clodronate-loaded liposomes), and two mouse models (macrophage Fas-induced apoptosis and diphtheria toxin receptor activation specifically in monocytes and macrophages), reduce SDF-1 in the BM and promote HSC mobilization to the PB [144-146]. Even though both BM monocytes and macrophages express SDF-1, hematopoietic cell-derived SDF-1 does not affect HSCs

chemotaxis [98,99].

Macrophages and monocytes promote HSCs quiescence

A subpopulation of BM machophages and monocytes secrete prostaglandin E2, which upregulates SDF-1 expression in the BM, CXCR4 in HSPCs and limits the production of reactive oxygen species (ROS) in HSCs, all essential for HSCs quiescence [147].

Macrophages regulate HPCs chemotaxis, releasing immature erythroid cells from the BM

Bone marrow macrophages are in intimate contact with erythroblasts, and its selective depletion mobilizes immature erythroid cells to the PB [148].

Neurons also modulate the BM microenvironment and hematopoiesis

Since the 17th century, it is known that the BM is richly innervated, however, the neural role in BM hematopoiesis has only very recently been scrutinized [4,149]. Neuronal activity indirectly regulates hematopoiesis, by modulating the BM microenvironment.

Neurons regulate HSCs chemotaxis, releasing HSCs from the BM

Noradrenaline-producing neurons activate β2- and β3- adrenergic receptors in osteoblasts and MSCs, which decrease BM SDF-1 expression, thereby mobilizing HSCs to the PB, both in homeostasis (circadian rhythmicity) [149,150] and induced mobilization (G-CSF-induced) models [151,152].

Nonmyelinating Schwann cells

Bone marrow neuron-wrapping nonmyelinating Schwann cells pro-

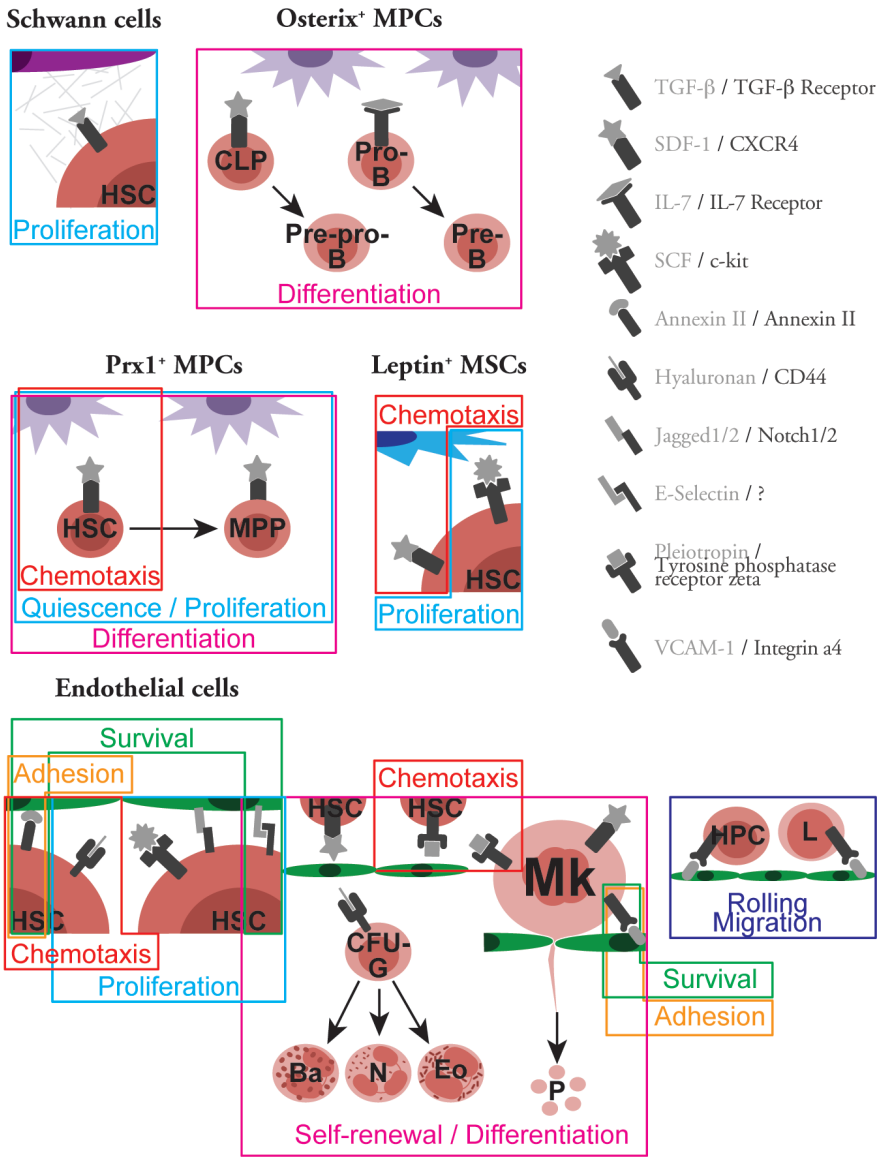


Figure 1.5. The hematopoietic cues in the niche: the role of Schwann cells, Osterix⁺ mesenchymal progenitor cells (MPCs), Prx1⁺ MPCs, Leptin⁺ mesenchymal stem cells (MSCs) and endothelial cells. **HSC**, hematopoietic stem cell. **CLP**, common myeloid progenitor. **Pre-pro-B**, early B lymphocyte progenitor. **Pro-B**, B lymphocyte progenitor **Pre-B**, late B lymphocyte progenitor. **MPP**, multipotent progenitor. **CFU-G**, colony forming untit-granulocyte. **Ba**, basophil. **N**, neutrophil. **Eo**, eosinophil. **Mk**, megakaryocyte. **HPC**, hematopoietic progenitor cell. **L**, lymphocytes.

mote HSC quiescence (Figure 1.5).

Nonmyelinating Schwann cells promote HSCs quiescence

Transforming growth factor-β (TGF-β)/Smad signaling in HSCs maintains its dormancy. Nonmyelinating Schwann cells activate latent transforming growth factor-β in the BM, which is the limiting step for TGF-β signaling. Transforming growth factor-β signaling abrogation in TGF-β receptor $II^{-/-}$ mice leads to reduced BM LT-HSCs and increased HSCs proliferation [100,153,154] (Figure 1.5).

Mesenchymal stem/progenitor cells

There are at least 2 types of stem cells in the BM; HSCs, the source of hematopoiesis (and main focus of this Thesis), and MSCs, which give rise to osteogenic and adipogenic cells. Several types of mesenchymal stem/progenitor cells have been identified: $Osterix^+$ MPCs, $Prx1^+$ MPCs, $MCAM^+$/$Nestin^+$ MSCs and Leptin $receptor^+$ MSCs. Whether these cells belong to the same cell type is not yet clear, so they will be presented independently.

$Osterix^+$ MPCs

Osterix is a transcription factor selectively expressed by non-self-renewing osteoprogenitors (MPCs) and mature osteoblasts and osteoclasts [98,155,156]. $Osterix^+$ MPCs are required for normal hematopoiesis, as disturbance of $Osterix^+$ cells (in $OsterixCre\text{-}Dicer^{f/f}$ and in $OsterixCre\text{-}Sdbs^{f/f}$ mice) is sufficient to trigger myelodysplasia (abnormal BM hematopoiesis) and secondary leukemia [157]. $Osterix^+$ MPCs are required for B lymphopoiesis.

$Osterix^{\pm}$ MPCs regulate HPCs differentiation

$Osterix^+$ cell-derived SDF-1 was recently shown to be required for the differentiation of early B lymphocyte precursors, as $Osterix\text{-}CreSDF1^{fl/-}$

mice exhibit normal BM CLP numbers, but reduced pre-pro B lymphocytes [98].

Specific Osterix$^+$ MPC disturbance (either by microRNA abrogation – in OsterixCre-Dicer$^{f/f}$ and OsterixCre-Sdbs$^{f/f}$ mice – or G protein-coupled receptors – in OsterixCre-$G_s\alpha^{f/f}$ mice) induces striking BM and PB B lymphocyte decrease, due to the reduction of the pre-B to pro-B transition, triggered by IL-7 reduction in the BM [157-159].

The ablation of niche-specific SDF-1 production in OsterixCre-SDF-1$^{fl/-}$ mice leads to a reduction in BM pre-pro B lymphocytes, without changes in the more primitive precursors [98] (Figure 1.5).

Prx1$^+$ MPCs

Prx1 is a transcription factor selectively expressed by renewing osteoprogenitors and mature osteoblasts and osteoclasts [98]. Some Prx1$^+$ cells are Leptin receptor$^+$ perivascular cells, and Prx1 is a more primitive MPC marker relative to Osterix [98,99]. Prx1$^+$ cells are required for HSCs chemotaxis and B lymphopoiesis.

Prx1$^\pm$ MPCs regulate HSCs chemotaxis, retaining HSCs in the BM

Stromal cell-derived factor-1 is the most potent HSC chemoattractant, which is mainly expressed by Prx1$^+$ MPCs and Leptin receptor$^+$ perivascular MSCs (mentioned below), but also by Osterix$^+$ MPCs, endothelial cells and osteoblasts [98,99]; SDF-1 concentration is typically higher in the BM than in the PB, such that when SDF-1 gradient is inverted (either by a decrease in SDF-1 availability in the BM, or an increase in PB SDF-1), HSCs mobilize to the periphery, while increasing BM SDF-1 enhances HSC engraftment [122,134,135,160-164]. Treatment of HSPCs with the SDF-1 receptor CXCR4 neutralizing antibody prevents its engraftment in a BMT setting, and transplantation of CXCR4-overexpressing cells (induced by SCF and IL-6 treatment) or transplantation CXCR4-functionally expressing cells (cells which migrated in response to an

SDF-1 gradient) enhances HSCs engraftment [160]. Prx1^{+} MPCs-derived SDF-1 promotes HSCs maintenance in the BM, as Prx1CreSDF-1$^{fl/fl}$ and Prx1CreSDF-1$^{fl/-}$ mice exhibit reduced BM HSCs and increased spleen HSCs [98,99] (Figure 1.5).

Prx1$^{\pm}$ MPCs promote HSCs quiescence

Genetic deletion of CXCR4 (in MxCre-CXCR4$^{f/-}$ mice) results in severe reduction of HSC levels and exit from the quiescent G_0 phase of the cell cycle [162]. Likewise, induced SDF-1 cell depletion (in SDF-1-DTR mice) severely diminishes SDF-1 and SCF in the BM, reduces HSCs number and size and induces HSC quiescence [165]. Prx1^{+} MPCs-derived SDF-1 promotes HSCs quiescence in the BM, as BM HSCs in Prx1CreSDF-1$^{fl/fl}$ and Prx1CreSDF-1$^{fl/-}$ mice are more actively cycling [98] (Figure 1.5).

Prx1$^{\pm}$ MPCs regulate HPCs differentiation

Prx1^{+} cell-derived SDF-1 is required for B lymphopoiesis, as Prx1CreSDF-1$^{fl/fl}$ and Prx1CreSDF-1$^{fl/-}$ mice exhibit reduced BM B lymphocytes, from the MPP stage of differentiation to the most mature B lymphocytes [98,99]. In fact, induced SDF-1 cell depletion (SDF-1-DTR mice) promotes myelopoiesis, and both SDF-1-abundant reticular cell depletion and deletion of CXCR4 reduce the proliferation of lymphoid progenitors, severely reducing the numbers of B lymphocytes in the BM [162,165] (Figure 1.5).

MCAM^{+}/Nestin MSCs

Human BM MCAM^{+} and mouse BM Nestin^{+} cells are perivascular MSCs. Human MCAM^{+} MSCs are capable of recapitulating the BM microenvironment in ectopic sites, through bone production, recruitment of neighboring blood vessels and blood-derived HSCs, and support HSCs maintenance [125,166]. Murine BM Nestin^{+} MSCs are closely associated with HSCs and adrenergic nerve fibers, and highly express HSC maintenance genes (SDF-1, SCF, Angpt1, IL-7, VCAM-1 and

Spp1); Nestin^{+} cell depletion diminishes HSCs in the BM and increases extramedullary hematopoiesis [28] (Figure 1.5).

Leptin receptor^{+} MSCs

Bone marrow leptin receptor-expressing cells are mesenchymal stem, perivascular cells.

Leptin receptor$^{\pm}$ MSCs regulate HSCs chemotaxis, retaining HSCs in the BM

As previously mentioned, SDF-1 is the most potent HSC chemoattractant. Leptin receptor^{+} cell-derived SDF-1 is required to retain HSCs and HPCs in the BM, as Leptin receptorCre-SDF-1$^{fl/fl}$ mice exhibit increased HSPCs in blood and spleen [99] (Figure 1.5).

Leptin receptor$^{\pm}$ MSCs promote HSCs proliferation

Together with ECs, leptin receptor^{+} MSCs are the major producers of SCF, which receptor, c-kit, is one of the major HSC markers [14,50,104,156,167-173]. Stem cell factor signaling via c-kit is critical for HSC proliferation; conditional deletion of either SCF or c-kit results in hematopoietic failure with decreased BM HSC content, and induced EC-specific SCF expression *in vitro* expands HSC numbers [101,174] (Figure 1.5).

Endothelial cells

Endothelial cells are the luminal vessel-lining cells. Besides the typical blood vessels found in any organ (arteries, capillaries and veins), the BM is richly vascularized by specific structures termed sinusoids [175,176]. The sinusoidal endothelium, also found in the liver and spleen, is composed of thin-walled, highly permeable fenestrated cells [110,175-178].

As shown above, several osteoblastic features do not concur with Schofield's theory. Likewise, the ECs (major components of the "vascular niche") do not only promote chemotaxis, adhesion, and differentiation

of HSPCs, but also HSCs proliferation and self-renewal, particularly in situations of BM stress (such as irradiation or chemotherapy). Since 2008, the discoveries in this field have been particularly intense, with accumulating evidence supporting the existence of a perivascular niche for HSCs (as mentioned in the beginning of this section), but also an EC-specific modulation of HSC proliferation. Recently, Rafii's laboratory proposed the existence of "angiocrine" genes, EC-derived growth factors and trophogens, which in the BM support hematopoiesis by regulating HSC quiescence versus self-renewal / proliferation and differentiation [179,180].

In this Thesis I will focus on EC-specific cues such as "angiocrine" factors, and will provide evidence on how these may regulate hematopoiesis.

Endothelial cells regulate HSCs chemotaxis, retaining HSCs in the BM

Endothelial cells and osteoblasts express annexin II, which through annexin II receptor promote HSCs engraftment, as previously referred [115].

Hyaluronan is a matrix component, mainly expressed by ECs, MSCs and HSPCs in the BM, whose receptor (cluster of differentiation 44, CD44) is expressed by HSCs, that promotes HSCs engraftment. Hyaluronan blockade (either therapeutic or genetic) reduces HSCs proliferation and BM content, while its increase, through *in vivo* administration, enriches BM ECs and macrophages with hyaluronan and increases overall BM, HSCs and HPCs counts [181-183].

Pleiotrophin, a heparin-binding growth factor expressed by BM ECs, inactivates the receptor protein tyrosine phosphatase receptor zeta in HSCs, promoting HSCs homing in the BM; Pleiotrophin$^{-/-}$ mice and anti-Pleiotrophin antibody treatment decreases BM HSC content, homing and retention in the BM following myelosuppression, whereas

the increase of Pleiotrophin signaling in tyrosine phosphatase receptor zeta$^{-/-}$ mice increases BM HSC content [184,185] (Figure 1.5).

Endothelial cells promote HSCs adhesion

Annexin II/annexin II receptor signaling in HSCs promote HSCs engraftment, as mentioned earlier [115] (Figure 1.5).

Endothelial cells promote HSCs survival

Annexin II receptor promotes HSCs survival, as mentioned earlier [115].

Endothelial selectin (E-selectin) is solely expressed by ECs in the BM and reduces HSC survival during BM recovery through a yet unidentified ligand (possibly ESL-1 or a glycosphingolipid); E-selectin$^{-/-}$ mice or E-selectin antagonist administration in mice enhance HSCs survival after chemotherapy or irradiation [172,186,187] (Figure 1.5).

Endothelial cells regulate HSCs quiescence/proliferation

Hyaluronan promotes HSCs proliferation and self-renewal. Hyaluronan blockade (either therapeutic or genetic) reduces HSCs proliferation and BM content, while its increase, through *in vivo* administration, enriches BM ECs and macrophages with hyaluronan and increases overall BM, HSCs and HPCs counts [181,183].

Jagged1/2 expressed by BM sinusoidal ECs (SECs) promotes proliferation and prevents exhaustion of LT-HSCs; during BM recovery post-irradiation, HSCs, in which Notch1/2 are active, are located near SECs. Bone marrow SECs disruption, through anti-VE-Cadherin plus anti-vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) antibody administration *in vivo*, severely impairs Jagged1/2 expression in SECs, followed by leukopenia and thrombocytopenia, completely disrupting hematopoietic recovery [104].

Endothelial selectin promotes PSGL1^{+} HSCs proliferation; E-selectin$^{-/-}$

mice or E-selectin antagonist administration in mice promote HSCs quiescence [172,186].

As previously indicated ECs are, together with Leptin^{+} perivascular cells, one of the major producers of SCF, which promote HSC maintenance and proliferation. Conditional deletion of either SCF or c-kit (in Tie2CreSCF$^{fl/-}$ mice and c-kit$^{W/\Delta GFP}$-derived HSPCs) results in hematopoietic failure with decreased BM HSC content and HSC failure to proliferate *in vitro*, and induced EC-specific SCF expression *in vitro* expands HSC numbers [101,174] (Figure 1.5).

Endothelial cells regulate HSPCs differentiation

Endothelial cell-derived SDF-1 prevents premature HSCs differentiation, as assessed in Tie2CreSDF-1$^{fl/fl}$ mice which exhibit reduced BM HSCs frequency, without changes in HSCs apoptosis or proliferation, and reduced donor-derived cells in competitive transplant assays [98,99]. Besides expressing SDF-1, BM ECs also translocate SDF-1 by from the circulation to the BM, where SDF-1 promotes megakaryopoiesis; transendothelial migration of CXCR4^{+} megakaryocytes promotes platelet production [122,134,188-190].

Endothelial selectin (E-selectin) promotes P-selectin glycoprotein 1 (PSGL1)$^{+}$ HSCs differentiation; E-selectin$^{-/-}$ mice or E-selectin antagonist administration in mice increase HSCs self-renewal [172,186].

Pleiotrophin, a heparin-binding growth factor expressed by BM ECs, inactivates the receptor protein tyrosine phosphatase receptor zeta in HSCs, promoting BM HSCs self-renewal; Pleiotrophin$^{-/-}$ mice and anti-Pleiotrophin antibody treatment decreases BM HSC content, impairs hematopoietic regeneration following myelosuppression and homing and retention in the BM, whereas the increase of Pleiotrophin signaling in tyrosine phosphatase receptor zeta$^{-/-}$ mice increases BM HSC content [185].

Vascular cell adhesion protein 1 is reported to mediate lymphocyte and HPC rolling and migration to the BM through the ECs, and megakaryocyte adhesion and transmigration (necessary for platelet release); Tie-2^{Cre}VCAM-1^{lox} mice exhibit marked reductions of immature B cells in the BM with correspondent increased numbers in the PB, and anti-VE-Cadherin treatment reduce EC VCAM-1 expression and impairs megakaryopoiesis [122,186,191-193].

Hyaluronan promotes myelopoiesis. Hyaluronan blockade (either therapeutic or genetic) reduces myelopoiesis, while its increase, through *in vivo* administration, enriches BM ECs and macrophages with hyaluronan and increases myelopoiesis, lymphopoiesis and megakaryopoiesis during hematopoietic recovery [181,183,194] (Figure 1.5).

Interestingly, there is a cross-talk between hematopoietic cells and the BM vessels; as shown above, BM ECs modulate hematopoiesis, but hematopoietic cells also modulate BM ECs. Megakaryocyte-derived thrombospondins negatively regulate angiogenesis [195]. Furthermore, VEGF, which is expressed by BM megakaryocytes, myeloid cells, erythrocytes and osteoblasts, is a major regulator of EC differentiation, proliferation, sprouting and permeability [196]. In addition, hyaluronan, which is expressed by HSPCs, MSCs and ECs, maintains vascular integrity [197].

Consistent with the critical role of BM ECs in BM recovery, several studies have shown that a BM with compromised vasculature is unable to recover, and it has long been known that the vascular area increases in a BM with high hematopoietic activity [19,110,178]. However, an increase of BM vessels does not always correspond to general hematopoietic changes: thrombospondin$^{-/-}$ mice show increased BM vessel coverage and increased megakaryopoiesis, but otherwise normal hematopoiesis [195]. Thus, hematopoiesis is dependent upon the surrounding molecular, and also cellular, cues.

MAJOR SIGNALING PATHWAYS DRIVING HEMATOPOIESIS

The orchestration of hematopoiesis has been introduced both at the macro and microenvironmental perspective. However, it is the activation of signaling pathways in the hematopoietic cells that underlies hematopoiesis, and the cross-talk between hematopoietic cells and its surroundings.

There are four main signaling pathways necessary for HSCs maintenance: Hedgehog (Hh), Notch, Wnt and HIF [87,198] (Table 1.2).

Table 1.2. Major macroenvironmental cues that perturb hematopoiesis.

	Survival	Quiescence / Proliferation	Self-renewal / Differentiation
Hh	—	Proliferation [199,200]	Differentiation [200]
Notch	Survival [119]	Proliferation [104,119,201-205]	Self-renewal [104,119,202-207]
Wnt	Survival [208]	Noncanonical pathway: Quiescence [120]; Canonical pathway: Proliferation [120,209]	Self-renewal [208,210,211]
HIF	—	Quiescence and HPC proliferation [80,83,84,86,87]	—

Hedgehog, Notch and Wnt are long recognized for its role in HSCs biology and hematopoietic development, however, its importance for adult hematopoiesis was recently challenged [212-216]. The data supporting Hh, Notch and Wnt involvement in several stages of hematopoiesis is abundant and consistent, and the studies suggesting its redundancy in hematopoiesis do not consider the noncanonical signaling pathways or the cross-talk between different signaling pathways. An excellent example is the Wnt signaling pathway, which had been under intense debate over the past few years, until in 2012 a paper by Sugimura et al. reported opposing roles of the canonical and noncanonical pathway in HSC self-renewal vs. proliferation [120,216]. Furthermore, signal integration is also critical in complex systems such as hematopoiesis: Wnt-mediated HSC self-renewal is Notch dependent, and the HIF-mediated HSC maintenance in an undifferentiated cell state requires Notch [207,217].

As previously stated, the role of macroenvironmental hypoxia is described in erythropoiesis, but not in HSC biology. Interestingly, it has been recently proposed that HSCs live in hypoxic niches to escape the production of potential destructive reactive oxygen species (ROS) and loss of quiescence, while ROS production in BM ECs, increased by irradiation, promote better engraftment in a BMT setting [84,86,114,218-220]. In fact, one of the problems of BM transplantation is precisely the increase of ROS in HSCs, which restricts its self-renewal capacity – opposed to HIF [87,221]. In fact, HIF-1α regulates HSCs quiescence in a dose-dependent manner, as assessed in $Mx1^{Cre}HIF\text{-}1\alpha^{lox}$, $Mx1^{Cre}VHL^{lox}$ and $Mx1^{Cre}HIF\text{-}1\alpha^{lox}VHL^{lox}$ mice [87].

Interestingly, of the signaling pathways shown to be involved in hematopoiesis regulation, Notch is the only that strictly requires cell-cell contact. This makes the Notch signaling pathway of particular interest for the definition of cell "niches" in the BM microenvironment during hematopoiesis. Furthermore, as discussed below, this pathway is also critical for angiogenesis (the formation of new blood vessels). We have thus focused on the role of Notch signaling pathway in the cross-talk between hematopoietic cells and ECs in the BM.

THE NOTCH SIGNALING PATHWAY

An overview

The Notch-Delta signaling pathway is an evolutionarily conserved signaling pathway that is required for embryonic development, regulation of tissue homeostasis, and maintenance of stem cells in adults. The Notch pathway has multiple roles in cell fate specification, tissue patterning, and morphogenesis through modulation of differentiation, proliferation, survival, and apoptosis [222-224]. In mammals, there are five canonical ligands, Delta-like 1 (Dll1), Delta-like 3 (Dll3), Delta-like 4 (Dll4), Jagged1 (Jag1), and Jagged-2 (Jag2), and four receptors, Notch1-4.

These ligands are type 1 cell-surface proteins with multiple tandem epidermal growth factor repeats in their extracellular domains, whereas Notch receptors are single-pass, type I transmembrane proteins [225].

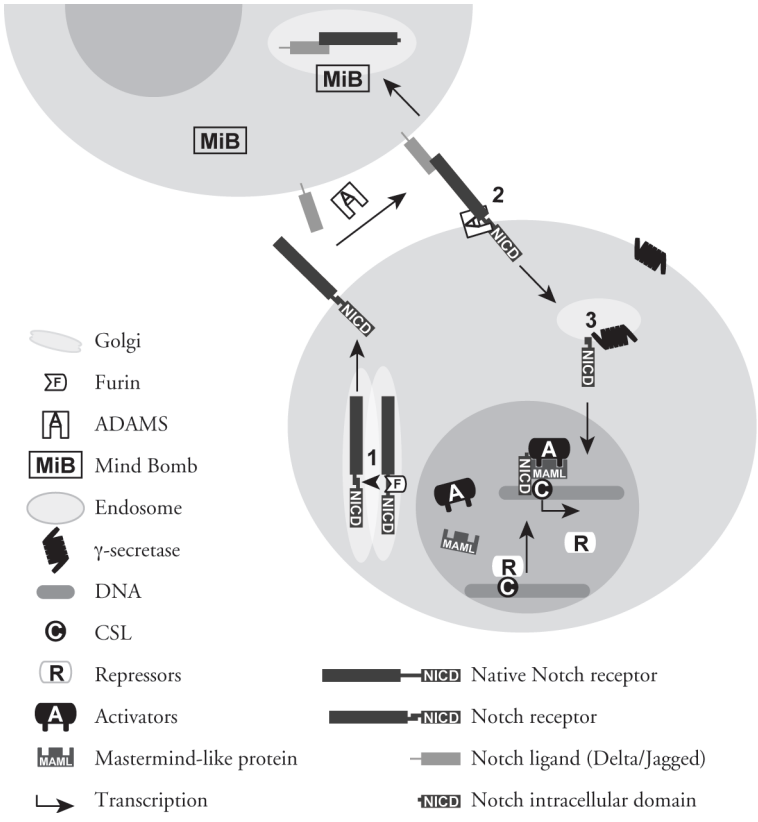


Figure 1.6. Schematic overview of the Notch signaling pathway.

Once the native Notch receptor protein is translated, it passes through the Golgi complex, where it may be modified by Fringe proteins (which modulate its affinity to particular Notch ligands), and is cleaved by furin-like convertases, transforming it to a transmembrane heterodimeric protein (Figure 1.6). A neighboring cell's Notch ligand binding to the extracellular domain of Notch receptor (NECD) triggers further proteolytic cleavages of Notch, first by a member of the a disintegrin and metalloprotease (ADAM) family within the juxtamembrane region, then by γ-secretase within the transmembrane domain. The final cleavage releases the Notch intracellular domain (NICD) from the cell membrane, which translocates to the nucleus and directly interacts with the transcription factor CSL (CBF1/RBPjκ/Su(H)/Lag-1). In the absence of NICD, CSL represses transcription through interactions with a transcriptional corepressor complex. Following Notch receptor activation, NICD binds to CSL thereby displacing the corepressor complex, and is recognized

by the transcription coactivator Mastermind-like (Maml). This triprotein complex recruits additional coactivators to activate transcription of downstream target genes [225] (Figure 1.6). Notch target genes are transcriptional repressors or derepressors, and include the helix-loop-helix proteins Hairy/Enhancer of Split (Hes), Hes-related proteins (Hey/HRT/HERP), and Notch regulated ankyrin repeat protein (Nrarp) [226].

The Notch signaling pathway functions as a lateral inhibition mechanism. It consists in ligand transcription reduction in Notch signal-receiving cells, and ligand recycling and activation in signal-sending cells, thereby hampering neighboring cells to adopt the same cell fate [227,228].

Dll4 and angiogenesis

In healthy adult individuals most blood vessel endothelial-lining cells proliferate at a very low rate [229]. In response to specific stimuli ECs can be activated, and proliferate, migrate, differentiate and mature into new stable blood vessels. This process of capillary formation from pre-existing ones is termed angiogenesis and is necessary for various physiological processes such as organ growth, wound healing, revascularization of ischemic tissue, ovulation, menstruation, implantation and pregnancy. Physiologic angiogenesis is a tightly regulated process, i.e. turned on for brief periods and then inhibited. Abnormalities in the molecular processes regulating angiogenesis can lead to pathologies as a result of excessive/abnormal angiogenesis (ex. diabetic retinopathy; hemangioma formation) or insufficient angiogenesis (ex. chronic wounds, tissue ischemia) [230,231]. A fine regulation of angiogenesis occurs through multiple molecular pathways that regulate positively (pro-angiogenic) and negatively (anti-angiogenic) angiogenesis (termed “angiogenesis balance”). Angiogenic models as the one herein presented are mainly based in developmental studies, murine postnatal retinal vasculature analysis and tumor growth.

Hypoxia is a major regulator of angiogenesis; in fact, the downstream

targets of HIF-1α include VEGF-A, its receptor VEGFR2 and the Notch ligand Dll4 [232,233]. Briefly, VEGF-A generates a gradient that, concomitantly with the Notch signaling, determines the formation of endothelial "tip" and "stalk" cells [234] (Figure 1.7A). The differentiated tip cell exhibits numerous filopodia and shows upregulation of membrane type 1-matrix metalloproteinase that degrades the vessel basement membrane and invades the surrounding matrix [234,235]. Concomitantly VEGF-A, along with other EC mitogenic factors produced by hypoxic cells or released from the extracellular matrix by proteolytic cleavage, induces stalk EC proliferation, resulting in the formation of a new vessel sprout. As the new sprout is established and perfused, VEGF levels decline and the vessel maturation initiates with the recruitment of pericytes and vascular smooth muscle cells around the newly formed vessel, which provide stabilization and maturation signals. Consequently adjoining ECs held together by tight and adherens junctions, seal the vessel lumen [236]. An extracellular matrix is then produced by ECs and surrounding cells, providing structural support and maintaining ECs quiescent, partly by laminin-induced Dll4 and Notch signaling [237,238].

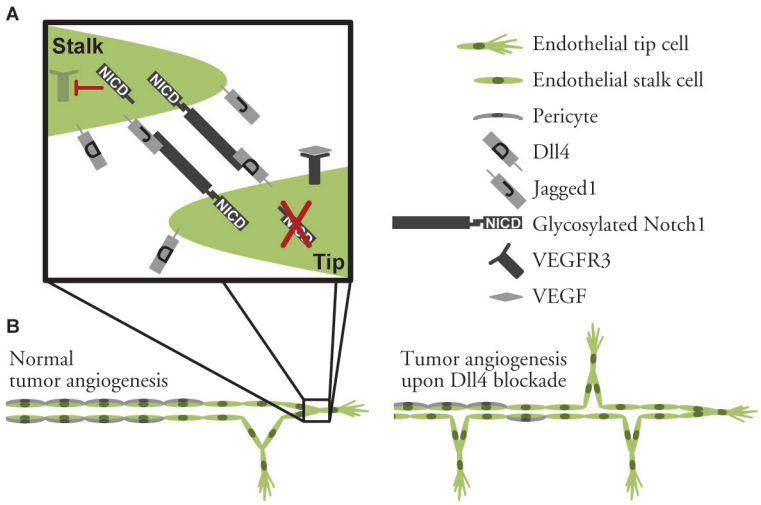


Figure 1.7. Dll4 regulates angiogenesis.
A. Dll4, Jagged1 and Notch1 regulate the tip cell phenotype. **B.** Dll4 blockade in tumor models increases vascular proliferation and tip cell phenotype, enhanced angiogenic sprouting and branching, with the production of thin caliber vessels, markedly reduced vessel lumens and reduced pericyte recruitment.

The emergence of endothelial tip cells during angiogenesis is tightly regulated by Dll4 [239-242]. Vascular endothelial growth factor signaling

enhances Dll4 expression in tip cells [243] (Figure 1.7A). Delta-like 4 then activates Notch1 and Notch4 receptors in adjacent stalk cells [244]. Notch activation in stalk ECs inhibits vessel filopodia formation and EC proliferation, by increasing VEGFR1 (a decoy receptor that negatively regulates angiogenesis) and decreasing VEGFR3 and possibly VEGFR2 and Nrp-1 [245-247]. This EC heterogeneity is tightly controlled by Notch glycosylation (through Fringe proteins – see above and Figure 1.6), which modifies Notch such that its functional affinity for Dll4 is enhanced, and Jagged1 bound to these receptors does not trigger Notch activation, thus antagonizing the pathway [248].

Interestingly, Dll4 emerged as an EC-specific Notch ligand crucial for artery specification [244,249]. Dll4 homozygous knockout mice are embryonic lethal, and depending on the strain, Dll4 heterozygous mice can be from fully lethal to 60% lethal – such a strong penetrant heterozygous embryonic lethality is comparable only with VEGF [250]. Table 1.3 reports the major embryonic defects of $Dll4^{-/-}$ and $Dll4^{+/-}$ mice [239,241,251].

Table 1.3. Dll4 is crucial for developmental angiogenesis.

	$Dll4^{-/-}$	$Dll4^{+/-}$
Viability	Lethal at E10.5 (formation of vascular network)	Viable (21%)
Embryo size	—	↓ in 69% of embryos – E9.5
Somite number	—	↓ in 69% of embryos – E9.5
Dorsal aorta	↓ diameter – E8.75 ↓↓ diameter – E9.0 Absent or highly reduced – E9.5	↓ diameter in 60% of embryos E9.0 → E9.5 → E10.0 Constriction in 90% of embryos E9.5 → E10.5
Anterior cardinal vein	↓ diameter – E8.75 ↓↓ diameter – E9.0 Absent or highly reduced – E9.5	↓ caliber – E9.5 Disorganization – E9.5
EC cell type	–	↑ tip cells

Several studies have documented the expression of Notch components in human and mouse tumor vessels, most notably Dll4. Delta-like 4 is strongly expressed in tumor blood vessels, compared to adjacent normal vessels [252-254]. The striking pattern of Dll4 expression in tumor vessels

prompted several groups to target Dll4-Notch activity and provided insight into a role for Dll4-Notch in regulating vascular sprouting. Local or systemic treatment with Dll4-Notch inhibitors in mice triggered the overgrowth of a non-functional tumor vasculature with consequent tumor growth inhibition and hypoxia increase in a variety of established human and rodent tumor models [255-257] (Figure 1.7B). In fact, Dll4 blockade was tested in various human and murine tumor cell lines grown subcutaneously in mice, with reduction of tumor growth from 50% to more than 90%, depending on the tumor model [255-257]. Delta-like 4 inhibition disrupts productive angiogenesis thereby impeding tumor growth [250]. Therapeutic target of Dll4 has thus raised major interest for tumor therapy development, such that there are two ongoing clinical trials for solid tumors [258,259].

Interestingly, Dll4 signaling does not trigger the same angiogenic phenotypes in all vascular models, even within the same organ. While Dll4 blockade in postnatal retinal vascularization is VEGF-dependent and increases angiogenic sprouting and EC proliferation, resulting in the formation of an abnormally dense primary capillary plexus, Dll4 inhibition in postangiogenic blood vessel remodeling in a model of oxygen-induced retinopathy is independent of VEGF, regulates vessel regression and maintains blood flow, thus resulting in a rapid revascularization of the ischemic portions of the retina [242,243,260,261]. This illustrates the potential of Dll4 modeling in diverse physiological and pathological conditions.

Dll4 and hematopoiesis

Besides its role in angiogenesis, Dll4 also regulates hematopoiesis in a context-dependent manner.

As mentioned above, Notch activation promotes HSCs proliferation and self-renewal [104,119,201-205,207] (Table 1.2). Membrane-bound Dll4,

however, seems to promote HSCs quiescence and self-renewal. Culture of HSPCs with recombinant Dll4 increases proliferation of phenotypic HSPCs and colony-forming cells (HPCs), through Notch pathway activation [203,262,263]. On the other hand, co-culture of HSPCs with stromal cells overexpressing Dll4 increases the proliferation of CLPs and induces HSCs quiescence and self-renewal by Notch pathway activation [263,264] (Figure 1.8A).

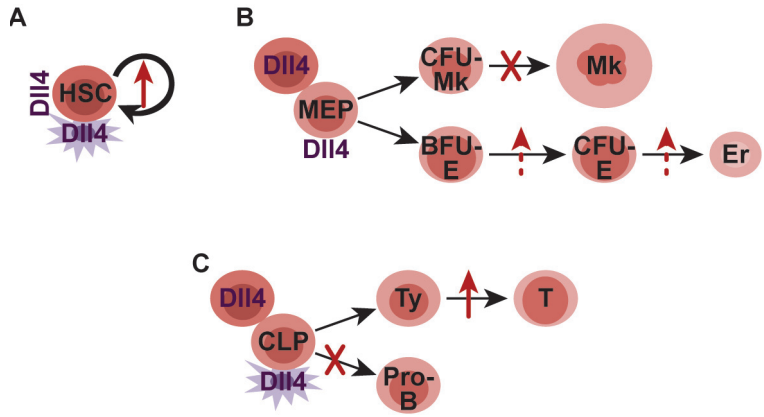


Figure 1.8. The role of Dll4 in hematopoiesis.
A. Dll4 enhances hematopoietic stem cells self-renewal.
B. Dll4 inhibits megakaryopoiesis and enhances erythropoiesis.
C. Dll4 promotes T lymphopoiesis and blocks B lymphopoiesis.
HSC, hematopoietic stem cell. **MEP**, megakaryocyte and erythrocyte progenitor. **CFU-Mk**, colony forming unit-megakaryocyte. **Mk**, megakaryocyte. **BFU-E**, burst forming unit-erythrocyte. **CFU-E**, colony forming unit-erythrocyte. **Er**, erythrocyte. **CLP**, common lymphoid progenitor. **Ty**, thymocyte. **T**, T lymphocyte. **Pro-B**, B lymphocyte progenitor.

Delta-like 4 is involved in megakaryo/erythroid differentiation, reducing megakaryocyte differentiation and eventually by promoting erythrocyte differentiation (Figure 1.8B). *In vitro* assays in which HSPCs are cultured with either recombinant Dll4 or stroma overexpressing Dll4 reveal a reduction in megakaryocytic differentiation in the most mature progenitors, while *in vivo* transplantation of hematopoietic cells overexpressing Dll4 elicits a profound thrombocytopenia without changes in the megakaryocyte progenitors, CFU-Mk [265-267]. The referred *in vitro* assays also revealed Dll4 has a modest effect in promoting erythrocytic differentiation; however, *in vivo* transplantation of Dll4-overexpressing hematopoietic cells does not modulate the erythrocytic parameters [265-267]. Interestingly, Dll4 is necessary for primitive erythropoiesis, shown by co-cultures of Dll4-overexpressing stroma cells and embryonic liver HSCs and Dll4$^{-/-}$ embryoid bodies assays [262,268] (Figure 1.8B).

Maeda's laboratory has recently revealed a role for Dll4 in the cross-talk between erythroblasts (late erythrocyte precursors) and HSCs. Hematopoietic stem cell self-renewal is maintained by erythroblasts in which Dll4 is repressed; the upregulation of Dll4 in erythroblasts (in $ErGFP^{Cre}Zbtb7a^{f/f}$ mice) promotes BM HSCs premature T lymphocyte differentiation [269].

Strikingly, Notch signaling is also a major regulator of lymphopoiesis, promoting T-cell differentiation at the expense of B cell proliferation, for which Dll4 seems to be crucial. Culture of HSCPs with stroma over-expressing Dll4 promotes T-cell differentiation and reduces B cell differentiation, and Dll4 blockade in thymic epithelial cells ($FoxN1^{Cre}Dll4^{lox/lox}$) blocks T-cell development at the "double negative" ($CD4^-CD8^-$) stage, corresponding to the most immature thymocytes, with accumulation of ectopic thymic B cells in a Notch1-dependent manner [270-272]. Remarkably, even though conditional Dll4 deletion in hematopoietic cells (and several stromal cells, $Mx1^{Cre}Dll4^{lox}$) does not affect lymphoid cells, induction of constitutive expression of Dll4 in hematopoietic cells (by viral infection *in vitro* followed by HSPC transplantation) promotes a lymphoproliferative disease, with ectopic "double positive" ($CD4^+CD8^+$) T-cells invasion, which progresses to a T-cell leukemia/lymphoma of non-transduced hematopoietic cells [265,266,270] (Figure 1.8C). This finding led us to question whether Dll4 blockade would interfere with leukemia cell function(s).

Malignant hematopoiesis: Leukemia

Leukemia is a term that defines a group of diseases which arise from malignant transformation of HSCs or their committed progenitors, and is triggered by sequential alterations in proto-oncogenes, tumor-suppressor genes, and microRNAs. These changes alter key regulatory processes in target cells by unleashing an unlimited capacity for self-renewal, subverting the control of normal proliferation, blocking cell differentiation,

and promoting resistance to apoptosis [273-278].

Leukemias are divided into 2 major groups, acute and chronic. In acute leukemias, there is a clinically relevant and evident onset of proliferation of leukemia cells, whereas in chronic leukemias, the same process can take years. Both groups are further divided into lymphoid or myeloid leukemias [42]. Like in normal hematopoiesis, leukemia onset, progression and treatment resistance are modulated by, and modulate, the microenvironment, including the BM vessels [279,280].

Notch signaling in Leukemia

Notch activating mutations are prevalent in approximately 50% of T-acute lymphoblastic leukemia (T-ALL) cases, establishing an important relationship between T-cell development and the induction of the disease [281-283]. In fact, Notch signaling disruption, by treatment with γ-secretase inhibitors, decrease cell viability and induce cell cycle arrest in different acute myeloid leukemia (AML), B- and T-ALL cell lines, and to a much lesser extent normal B lymphocytes [282,284-288].

Aggressive T-cell leukemia mouse models were established by Notch1 and Notch3 constitutive activation, and as previously mentioned, Dll4 overexpression in the neighboring hematopoietic cells [265,266,289,290], revealing that leukemogenesis may be favored by microenvironmental Dll4-mediated Notch activation. In fact, similar to solid tumor models, Dll4 blockade reduces solid leukemia growth *in vivo* (in AML and T-ALL cell lines), by affecting tumor angiogenesis and blocking Dll4/Notch3 signaling in the tumor cells [252,254-256,258,259,291,292]. An important relationship between endothelial-specific Dll4 levels and tumor dormancy was established in xenografts of T-ALL cell lines [254,291,293], and Dll4 overexpression in human AML BM is associated with higher angiogenesis and poor prognosis [294,295]. However, this relationship between higher Dll4 levels and leukemia onset and progression is still somewhat controversial, as co-culture of an AML cell line, HL-60, with a stromal cell line

overexpressing Dll4 promotes myeloid differentiation [296], resulting in cell cycle arrest and apoptosis [273,296].

AIMS

Hematopoiesis involves (and requires) cell-intrinsic and also micro- and macro-environmental signals and interactions. Targeting the BM microenvironment, or modulating the host macroenvironment, consequently perturbs hematopoiesis.

In this Thesis, we aimed at scrutinizing the crosstalk between ECs that comprise the BM vessels and hematopoietic cells in the BM, by using two different strategies – Dll4 blockade and chronic intermittent hypoxia –, and the cell-intrinsic involvement of Dll4:Notch signaling in leukemia cell survival and proliferation:

1. To characterize the role of Dll4 blockade in the BM microenvironment and on hematopoiesis following myelossupression (Chapter 2);

2. To characterize the direct effects of Dll4:Notch signaling blockade in leukemia cells (Chapter 3).

3. To characterize the effects of chronic intermittent hypoxia in BM microenvironment and on hematopoiesis (Chapter 4).

REFERENCES

1. DuVerney, M. De la structure et du sentiment de la moelle. *Académie royale des sciences* (1700).

2. Cooper, B. The origins of bone marrow as the seedbed of our blood: from antiquity to the time of Osler. *Proc (Bayl Univ Med Cent)* 24, 115–118 (2011).

3. Hooke, R. *Micrographia*. 1–379 (Royal Society of London, 1664).

4. Havers, C. *Osteologia Nova, Or Some New Observations of the Bones, and the Parts Belonging to Them*. (1691).

5. Mandl, L. *Anatomie microscopique*. (J. Baillière et Fils, 1857).

6. Robin, C. H. *Dictionnaire Encyclopédique des Sciences Médicales*. 61, 1–813 (Place de l'école-de-médicine, 1864).

7. Mazzarello, P., Calligaro, A. L. & Calligaro, A. Giulio Bizzozero: a pioneer of cell biology. *Nat Rev Mol Cell Biol* 2, 776–781 (2001).

8. Lorenz, E., Uphoff, D., Reid, I. R. & Shelton, E. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *J. Natl. Cancer Inst.* 12, 197–201 (1951).

9. Nowell, P. C., Cole, L. J., Habermeyer, J. G. & Roan, P. L. Growth and continued function of rat marrow cells in x-radiated mice. *Cancer Research* 16, 258–261 (1956).

10. Till, J. E.McCulloch. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* 14, 213–222 (1961).

11. Becker, A. J., McCulloch, E. A. & Till, J. E. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* 197, 452–454 (1963).

12. Siminovitch, L., McCulloch, E. A. & Till, J. E. The distribution of colony-forming cells among spleen colonies. *J. Cell. Physiol.* 62, 327–336 (1963).

13. Wu, A. M., Till, J. E., Siminovitch, L. & McCulloch, E. A. Cytological evidence for a relationship between normal hemotopoietic colony-forming cells and cells of the lymphoid system. *J. Exp. Med.* 127, 455–464 (1968).

14. Osawa, M., Hanada, K., Hamada, H. & Nakauchi, H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* 273, 242–245 (1996).

15. Thomas, E. D., Lochte, H. L., LU, W. C. & Ferrebee, J. W. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N. Engl. J. Med.* 257, 491–496 (1957).

16. Appelbaum, F. R. *Hematopoietic-cell transplantation at 50. N. Engl. J. Med.* 357, 1472–1475 (2007).

17. Gratwohl, A. *et al.* Hematopoietic stem cell transplantation: a global perspective. *JAMA* 303, 1617–1624 (2010).

18. Drummond, W. B. & Muir, R. On the Structure of the Bone-Marrow in Relation to Blood-Formation. *J Anat Physiol* 28, 125–141 (1893).

19. Fliedner, T. M., Bond, V. P. & Cronkite, E. P. Structural, cytologic and autoradiographic (H3-thymidine) changes in the bone marrow following total body irradiation. *Am. J. Pathol.* 38, 599–623 (1961).

20. Lord, B. I. & Hendry, J. H. The distribution of haemopoietic colony-forming units in the mouse femur, and its modification by x rays. *Br J Radiol* 45, 110–115 (1972).

21. Lord, B. I., Testa, N. G. & Hendry, J. H. The relative spatial distributions of CFUs and CFUc in the normal mouse femur. *Blood* 46, 65–72 (1975).

22. Schofield, R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* 4, 7–25 (1978).

23. Nilsson, S. K., Johnston, H. M. & Coverdale, J. A. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. *Blood* 97, 2293–2299 (2001).

24. Kiel, M. J. *et al.* SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 121, 1109–1121 (2005).

25. Ellis, S. *et al.* The histo-morphometric relationship between bone, blood vessels and hemopoietic stem cells. *MAM* 14, (2008).

26. Xie, Y. *et al.* Detection of functional haematopoietic stem cell niche using real-time imaging. *Nature* 457, 97–102 (2008).

27. Winkler, I. G. *et al.* Positioning of bone marrow hematopoietic and stromal cells relative to blood flow in vivo: serially reconstituting hematopoietic stem cells reside in distinct nonperfused niches. *Blood* 116, 375–385 (2010).

28. Méndez-Ferrer, S. *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466, 829–834 (2010).

29. Ellis, S. L. *et al.* The relationship between bone, hemopoietic stem cells, and vasculature. *Blood* 118, 1516–1524 (2011).

30. Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C. & Keller, G. A common precursor for hematopoietic and endothelial cells. *Development* 125, 725–732 (1998).

31. Jaffredo, T., Gautier, R., Eichmann, A. & Dieterlen-Lièvre, F. Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development* 125, 4575–4583 (1998).

32. Nishikawa, S. I. *et al.* In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. *Immunity* 8, 761–769 (1998).

33. Zovein, A. C. *et al.* Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell* 3, 625–636 (2008).

34. Lancrin, C. *et al.* The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature* 457, 892–895 (2009).

35. Eilken, H. M., Nishikawa, S.-I. & Schroeder, T. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature* 457, 896–900 (2009).

36. Chen, M. J. *et al.* Erythroid/Myeloid Progenitors and Hematopoietic Stem Cells Originate from Distinct Populations of Endothelial Cells. *Stem Cell* 9, 541–552 (2011).

37. Hoffman, R. *et al. Hematology*. 1–3581 (Elsevier, 2005).

38. Costa, G., Kouskoff, V. & Lacaud, G. Origin of blood cells and HSC production in the embryo. *Trends in Immunology* 33, 215–223 (2012).

39. Shiozawa, Y. & Taichman, R. S. Getting blood from bone: An emerging understanding of the role that osteoblasts play in regulating hematopoietic stem cells within their niche. *Exp. Hematol.* 40, 685–694 (2012).

40. Brannan, D. Extramedullary hematopoiesis in anemias. *Bulletin of the Johns Hopkins Hospital* 41, 104–135 (1927).

41. Zhu, J. & Emerson, S. G. Hematopoietic cytokines, transcription factors and lineage commitment. *Oncogene* 21, 3295–3313 (2002).

42. *Concise Manual of Hematology and Oncology (Springer, 2008)*. 1–1021 (Springer, 2008).

43. Navarro, M. *et al.* Massive intrahepatic extramedullary hematopoiesis in myelofibrosis. *Abdom Imaging* 25, 184–186 (2000).

44. Koch, C. A., Li, C.-Y., Mesa, R. A. & Tefferi, A. Nonhepatosplenic extramedullary hematopoiesis: associated diseases, pathology, clinical course, and treatment. *Mayo Clin. Proc.* 78, 1223–1233 (2003).

45. Ogawa, M. Differentiation and proliferation of hematopoietic stem cells. *Blood* 81, 2844–2853 (1993).

46. Taichman, R. S. & Emerson, S. G. The role of osteoblasts in the hematopoietic microenvironment. *Stem Cells* 16, 7–15 (1998).

47. Fliedner, T. M., Graessle, D., Paulsen, C. & Reimers, K. Structure and function of bone marrow hemopoiesis: mechanisms of response to ionizing radiation exposure. *Cancer Biother. Radiopharm.* 17, 405–426 (2002).

48. Kopp, H.-G., Avecilla, S. T., Hooper, A. T. & Rafii, S. The bone marrow vascular niche: home of HSC differentiation and mobilization. *Physiology (Bethesda)* 20, 349–356 (2005).

49. Morrison, S. J. & Weissman, I. L. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* 1, 661–673 (1994).

50. Morrison, S. J., Wandycz, A. M., Hemmati, H. D., Wright, D. E. & Weissman, I. L. Identification of a lineage of multipotent hematopoietic progenitors. *Development* 124, 1929–1939 (1997).

51. Wu, L., Antica, M., Johnson, G. R., Scollay, R. & Shortman, K. Developmental potential of the earliest precursor cells from the adult mouse thymus. *J. Exp. Med.* 174, 1617–1627 (1991).

52. Matsuzaki, Y. *et al.* Characterization of c-kit positive intrathymic stem cells that are restricted to lymphoid differentiation. *J. Exp. Med.* 178, 1283–1292 (1993).

53. Kondo, M., Weissman, I. L. & Akashi, K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 91, 661–672 (1997).

54. Akashi, K., Traver, D., Miyamoto, T. & Weissman, I. L. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 404, 193–197 (2000).

55. Miyamoto, T. *et al.* Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. *Developmental Cell* 3, 137–147 (2002).

56. Seita, J. & Weissman, I. L. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med* 2, 640–653 (2010).

57. Manz, M. G., Traver, D., Miyamoto, T., Weissman, I. L. & Akashi, K. Dendritic cell potentials of early lymphoid and myeloid progenitors. *Blood* 97, 3333–3341 (2001).

58. Fisher, J. W. Landmark advances in the development of erythropoietin. *Exp. Biol. Med. (Maywood)* 235, 1398–1411 (2010).

59. Lawrence, J. S., Valentine, W. N. & Dowdy, A. H. The effect of radiation on hemopoiesis; is there an indirect effect? *Blood* 3, 593–611 (1948).

60. Jacobson, L. O., Simmons, D. L., Marks, E. K. & Eldredge, J. H. Recovery from radiation injury. *Science* 113, 510–511 (1951).

61. Till, J. E., McCulloch, E. A. & Siminovitch, L. A stochastic model of stem cell proliferation, based on the growth of spleen colony-forming cells. *Proc. Natl. Acad. Sci. U.S.A.* 51, 29–36 (1964).

62. Boggs, S. S., Chervenick, P. A. & Boggs, D. R. The effect of postirradiation bleeding or endotoxin on proliferation and differentiation of hematopoietic stem cells. *Blood* 40, 375–389 (1972).

63. Boggs, S. S. & Boggs, D. R. Effect of bleeding on hematopoiesis following irradiation and marrow transplantation. *Blood* 45, 205–212 (1975).

64. Cheshier, S. H., Prohaska, S. S. & Weissman, I. L. The effect of bleeding on hematopoietic stem cell cycling and self-renewal. *Stem Cells Dev.* 16, 707–717 (2007).

65. Richman, C. M., Weiner, R. S. & Yankee, R. A. Increase in circulating stem cells following chemotherapy in man. *Blood* 47, 1031–1039 (1976).

66. Abrams, R. A., McCormack, K., Bowles, C. & Deisseroth, A. B. Cyclophosphamide treatment expands the circulating hematopoietic stem cell pool in dogs. *J. Clin. Invest.* 67, 1392–1399 (1981).

67. Yeager, A. M., Levin, J. & Levin, F. C. The effects of 5-fluorouracil on hematopoiesis: studies of murine megakaryocyte-CFC, granulocyte-macrophage-CFC, and peripheral blood cell levels. *Exp. Hematol.* 11, 944–952 (1983).

68. Vetvicka, V., Kincade, P. W. & Witte, P. L. Effects of 5-fluorouracil on B lymphocyte lineage cells. *J. Immunol.* 137, 2405–2410 (1986).

69. Harrison, D. E. & Lerner, C. P. Most primitive hematopoietic stem cells are stimulated to cycle rapidly after treatment with 5-fluorouracil. *Blood* 78, 1237–1240 (1991).

70. Morrison, S. J., Wright, D. E. & Weissman, I. L. Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. *Proc. Natl. Acad. Sci. U.S.A.* 94, 1908–1913 (1997).

71. Baldridge, M. T., King, K. Y. & Goodell, M. A. Inflammatory signals regulate hematopoietic stem cells. *Trends in Immunology* 32, 57–65 (2011).

72. King, K. Y. & Goodell, M. A. Inflammatory modulation of HSCs: viewing the HSC as a foundation for the immune response. *Nat Rev Immunol* 11, 685–692 (2011).

73. de Bruin, A. M. *et al.* IFN induces monopoiesis and inhibits neutrophil development during inflammation. *Blood* 119, 1543–1554 (2012).

74. Travlos, G. Normal Structure, Function, and Histology of the Bone Marrow. *Toxicologic Path.* 34, 548–565 (2006).

75. Gomes, A. L., Carvalho, T., Serpa, J., Torre, C. & Dias, S. Hypercholesterolemia promotes bone marrow cell mobilization by perturbing the SDF-1:CXCR4 axis. *Blood* 115, 3886–3894 (2010).

76. Ferraro, F. *et al.* Diabetes impairs hematopoietic stem cell mobilization by altering niche function. *Science Translational Medicine* 3, 104ra101 (2011).

77. Reissmann, K. R. Studies on the mechanism of erythropoietic stimulation in parabiotic rats during hypoxia. *Blood* 5, 372–380 (1950).

78. Koller, M. R., Bender, J. G., Miller, W. M. & Papoutsakis, E. T. Reduced oxygen tension increases hematopoiesis in long-term culture of human stem and progenitor cells from cord blood and bone marrow. *Exp. Hematol.* 20, 264–270 (1992).

79. Kalra, V. K., Shen, Y., Sultana, C. & Rattan, V. Hypoxia induces PECAM-1 phosphorylation and transendothelial migration of monocytes. *Am. J. Physiol.* 271, H2025–34 (1996).

80. Cipolleschi, M. G., Sbarba, Dello, P. & Olivotto, M. The role of hypoxia in the maintenance of hematopoietic stem cells. *Blood* 82, 2031–2037 (1993).

81. Mostafa, S. S., Miller, W. M. & Papoutsakis, E. T. Oxygen tension influences the differentiation, maturation and apoptosis of human megakaryocytes. *Br. J. Haematol.* 111, 879–889 (2000).

82. Hevehan, D. L., Papoutsakis, E. T. & Miller, W. M. Physiologically significant effects of pH and oxygen tension on granulopoiesis. *Exp. Hematol.* 28, 267–275 (2000).

83. Ivanovi , Z. *et al.* Incubation of murine bone marrow cells in hypoxia ensures the maintenance of marrow-repopulating ability together with the expansion of committed progenitors. *Br. J. Haematol.* 108, 424–429 (2000).

84. Parmar, K., Mauch, P., Vergilio, J.-A., Sackstein, R. & Down, J. D. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5431–5436 (2007).

85. Levesque, J.-P. *et al.* Hematopoietic Progenitor Cell Mobilization Results in Hypoxia with Increased Hypoxia-Inducible Transcription Factor-1α and Vascular Endothelial Growth Factor A in Bone Marrow. *Stem Cells* 25, 1954–1965 (2007).

86. Kubota, Y., Takubo, K. & Suda, T. Bone marrow long label-retaining cells reside in the sinusoidal hypoxic niche. *Biochemical and Biophysical Research Communications* 366, 335–339 (2008).

87. Takubo, K. *et al.* Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. *Cell Stem Cell* 7, 391–402 (2010).

88. Weigert, A. *et al.* HIF-1α is a negative regulator of plasmacytoid DC development in vitro and in vivo. *Blood* 120, 3001–3006 (2012).

89. Jing, D. *et al.* Oxygen tension plays a critical role in the hematopoietic microenvironment in vitro. *Haematologica* 97, 331–339 (2012).

90. Jelkmann, W. Control of erythropoietin gene expression and its use in medicine. *Meth. Enzymol.* 435, 179–197 (2007).

91. Wang, G. L., Jiang, B. H., Rue, E. A. & Semenza, G. L. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc. Natl. Acad. Sci. U.S.A.* 92, 5510–5514 (1995).

92. Bruick, R. K. & McKnight, S. L. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294, 1337–1340 (2001).

93. Epstein, A. C. *et al.* C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107, 43–54 (2001).

94. Semenza, G. L. Hypoxia-Inducible Factors in Physiology and Medicine. *Cell* 148, 399–408 (2012).

95. Semenza, G. L. *et al.* Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J. Biol. Chem.* 271, 32529–32537 (1996).

96. Dexter, T. M., Allen, T. D. & Lajtha, L. G. Conditions controlling the proliferation of haemopoietic stem cells in vitro. *J. Cell. Physiol.* 91, 335–344 (1977).

97. Calvi, L. M. *et al.* Osteoblastic expansion induced by parathyroid hormone receptor signaling in murine osteocytes is not sufficient to increase hematopoietic stem cells. *Blood* 119, 2489–2499 (2012).

98. Greenbaum, A. *et al.* CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* (2013). doi:10.1038/nature11926

99. Ding, L. & Morrison, S. J. Haematopoietic stem

cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* (2013). doi:10.1038/nature11885

100. Yamazaki, S. *et al.* Nonmyelinating Schwann Cells Maintain Hematopoietic Stem Cell Hibernation in the Bone Marrow Niche. *Cell* 147, 1146–1158 (2011).

101. Ding, L., Saunders, T. L., Enikolopov, G. & Morrison, S. J. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 481, 457–462 (2012).

102. Taichman, R. S., Reilly, M. J. & Emerson, S. G. Human osteoblasts support human hematopoietic progenitor cells in vitro bone marrow cultures. *Blood* 87, 518–524 (1996).

103. El-Badri, N. S., Wang, B. Y., Cherry & Good, R. A. Osteoblasts promote engraftment of allogeneic hematopoietic stem cells. *Exp. Hematol.* 26, 110–116 (1998).

104. Butler, J. M. *et al.* Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell* 6, 251–264 (2010).

105. Chute, J. P. *et al.* Transplantation of vascular endothelial cells mediates the hematopoietic recovery and survival of lethally irradiated mice. *Blood* 109, 2365–2372 (2007).

106. Calvi, L. M. *et al.* Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 425, 841–846 (2003).

107. Visnjic, D. *et al.* Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood* 103, 3258–3264 (2004).

108. Zhang, J. *et al.* Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 425, 836–841 (2003).

109. Schepers, K., Hsiao, E. C., Garg, T., Scott, M. J. & Passegué, E. Activated Gs signaling in osteoblastic cells alters the hematopoietic stem cell niche in mice. *Blood* (2012). doi:10.1182/blood-2011-11-395418

110. Branemark, P. I. Experimental investigation of microcirculation in bone marrow. *Angiology* 12, 293–305 (1961).

111. Simsek, T. *et al.* The Distinct Metabolic Profile of Hematopoietic Stem Cells Reflects Their Location in a Hypoxic Niche. *Stem Cell* 7, 380–390 (2010).

112. Arai, F. *et al.* Tie2/Angiopoietin-1 Signaling Regulates Hematopoietic Stem Cell Quiescence in the Bone Marrow Niche. *Cell* 118, 149–161 (2004).

113. Celso, Lo, C. *et al.* Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature* 457, 92–96 (2009).

114. Lewandowski, D. *et al.* In vivo cellular imaging pinpoints the role of reactive oxygen species in the early steps of adult hematopoietic reconstitution. *Blood* 115, 443–452 (2010).

115. Jung, Y. *et al.* Annexin II expressed by osteoblasts and endothelial cells regulates stem cell adhesion, homing, and engraftment following transplantation. *Blood* 110, 82–90 (2007).

116. Nilsson, S. K. *et al.* Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood* 106, 1232–1239 (2005).

117. Stier, S. *et al.* Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *J. Exp. Med.* 201, 1781–1791 (2005).

118. Mazzon, C. *et al.* The critical role of agrin in the hematopoietic stem cell niche. *Blood* 118, 2733–2742 (2011).

119. Karanu, F. N. *et al.* The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. *J. Exp. Med.* 192, 1365–1372 (2000).

120. Sugimura, R. *et al.* Noncanonical Wnt signaling maintains hematopoietic stem cells in the niche. *Cell* 150, 351–365 (2012).

121. Sitnicka, E. *et al.* The effect of thrombopoietin on the proliferation and differentiation of murine hematopoietic stem cells. *Blood* 87, 4998–5005 (1996).

122. Avecilla, S. T. *et al.* Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nature Medicine* 10, 64–71 (2004).

123. Yoshihara, H. *et al.* Thrombopoietin/MPL Sig-

naling Regulates Hematopoietic Stem Cell Quiescence and Interaction with the Osteoblastic Niche. *Cell Stem Cell* 1, 685–697 (2007).

124. Qian, H. *et al.* Critical Role of Thrombopoietin in Maintaining Adult Quiescent Hematopoietic Stem Cells. *Cell Stem Cell* 1, 671–684 (2007).

125. Sacchetti, B. *et al.* Self-Renewing Osteoprogenitors in Bone Marrow Sinusoids Can Organize a Hematopoietic Microenvironment. *Cell* 131, 324–336 (2007).

126. Taichman, R. S. & Emerson, S. G. Human osteoblasts support hematopoiesis through the production of granulocyte colony-stimulating factor. *J. Exp. Med.* 179, 1677–1682 (1994).

127. Zhu, J. *et al.* Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells. *Blood* 109, 3706–3712 (2007).

128. Wilson, A. *et al.* c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. *Genes & Development* 18, 2747–2763 (2004).

129. Ivanova, N. B. *et al.* A stem cell molecular signature. *Science* 298, 601–604 (2002).

130. Kiel, M. J., Radice, G. L. & Morrison, S. J. Lack of Evidence that Hematopoietic Stem Cells Depend on N-Cadherin-Mediated Adhesion to Osteoblasts for Their Maintenance. *Cell Stem Cell* 1, 204–217 (2007).

131. Bromberg, O. *et al.* Osteoblastic N-cadherin is not required for microenvironmental support and regulation of hematopoietic stem and progenitor cells. *Blood* 120, 303–313 (2012).

132. Calvi, L. M. *et al.* Activated parathyroid hormone/parathyroid hormone-related protein receptor in osteoblastic cells differentially affects cortical and trabecular bone. *J. Clin. Invest.* 107, 277–286 (2001).

133. Adams, G. B. *et al.* Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature* 439, 599–603 (2005).

134. Heissig, B. *et al.* Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* 109, 625–637 (2002).

135. Kollet, O. *et al.* Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. *Nature Medicine* 12, 657–664 (2006).

136. Copp, D. H. & Cheney, B. Calcitonin—a Hormone from the Parathyroid which Lowers the Calcium-level of the Blood. *Nature* 193, 381–382 (1962).

137. Miyamoto, K. *et al.* Osteoclasts are dispensable for hematopoietic stem cell maintenance and mobilization. *Journal of Experimental Medicine* 208, 2175–2181 (2011).

138. Lymperi, S., Ersek, A., Ferraro, F., Dazzi, F. & Horwood, N. J. Inhibition of osteoclast function reduces hematopoietic stem cell numbers in vivo. *Blood* 117, 1540–1549 (2011).

139. Zimmer, S. N. *et al.* Crebbp haploinsufficiency in mice alters the bone marrow microenvironment, leading to loss of stem cells and excessive myelopoiesis. *Blood* 118, 69–79 (2011).

140. Gimble, J. M., Robinson, C. E., Wu, X. & Kelly, K. A. The function of adipocytes in the bone marrow stroma: an update. *Bone* 19, 421–428 (1996).

141. Naveiras, O. *et al.* Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature* 460, 259–263 (2009).

142. DiMascio, L. *et al.* Identification of adiponectin as a novel hemopoietic stem cell growth factor. *J. Immunol.* 178, 3511–3520 (2007).

143. Fujisaki, J. *et al.* In vivo imaging of Treg cells providing immune privilege

to the haematopoietic stem-cell niche. *Nature* 474, 216–219 (2011).

144. Winkler, I. G. *et al.* Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood* 116, 4815–4828 (2010).

145. Christopher, M. J., Rao, M., Liu, F., Woloszynek, J. R. & Link, D. C. Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice. *Journal of Experimental Medicine* 208, 251–260 (2011).

146. Chow, A. *et al.* Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *Jour-*

nal of Experimental Medicine 208, 261–271 (2011).

147. Ludin, A. *et al.* Monocytes-macrophages that express -smooth muscle actin preserve primitive hematopoietic cells in the bone marrow. *Nat. Immunol.* 13, 1072–1082 (2012).

148. Barbé, E., Huitinga, I., Döpp, E. A., Bauer, J. & Dijkstra, C. D. A novel bone marrow frozen section assay for studying hematopoietic interactions in situ: the role of stromal bone marrow macrophages in erythroblast binding. *Journal of Cell Science* 109 (Pt 12), 2937–2945 (1996).

149. Méndez-Ferrer, S., Lucas, D., Battista, M. & Frenette, P. S. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature* 452, 442–447 (2008).

150. Lucas, D., Battista, M., Shi, P. A., Isola, L. & Frenette, P. S. Mobilized hematopoietic stem cell yield depends on species-specific circadian timing. *Cell Stem Cell* 3, 364–366 (2008).

151. Katayama, Y. *et al.* Signals from the Sympathetic Nervous System Regulate Hematopoietic Stem Cell Egress from Bone Marrow. *Cell* 124, 407–421 (2006).

152. Méndez-Ferrer, S., Battista, M. & Frenette, P. S. Cooperation of 2- and 3-adrenergic receptors in hematopoietic progenitor cell mobilization. *Ann. N. Y. Acad. Sci.* 1192, 139–144 (2010).

153. Annes, J. P., Munger, J. S. & Rifkin, D. B. Making sense of latent TGFbeta activation. *Journal of Cell Science* 116, 217–224 (2003).

154. Larsson, J. & Karlsson, S. The role of Smad signaling in hematopoiesis. *Oncogene* 24, 5676–5692 (2005).

155. Nakashima, K. *et al.* The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108, 17–29 (2002).

156. Park, D. *et al.* Endogenous Bone Marrow MSCs Are Dynamic, Fate-Restricted Participants in Bone Maintenance and Regeneration. *Stem Cell* 10, 259–272 (2012).

157. Raaijmakers, M. H. G. P. *et al.* Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature* 464, 852–857 (2010).

158. Wu, J. Y. *et al.* Osteoblastic regulation of B lymphopoiesis is mediated by Gs{alpha}-dependent signaling pathways. *Proc Natl Acad Sci U S A* 105, 16976–16981 (2008).

159. Nagasawa, T. Microenvironmental niches in the bone marrow required for B-cell development. *Nat Rev Immunol* 6, 107–116 (2006).

160. Peled, A. *et al.* Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science* 283, 845–848 (1999).

161. Petit, I. *et al.* G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat. Immunol.* 3, 687–694 (2002).

162. Sugiyama, T., Kohara, H., Noda, M. & Nagasawa, T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 25, 977–988 (2006).

163. Slayton, W. B. *et al.* The role of the donor in the repair of the marrow vascular niche following hematopoietic stem cell transplant. *Stem Cells* 25, 2945–2955 (2007).

164. Jung, Y. *et al.* Hematopoietic stem cells regulate mesenchymal stromal cell induction into osteoblasts thereby participating in the formation of the stem cell niche. *Stem Cells* 26, 2042–2051 (2008).

165. Omatsu, Y. *et al.* The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity* 33, 387–399 (2010).

166. Corselli, M. *et al.* Perivascular support of human hematopoietic cells. *Blood* (2013). doi:10.1182/blood-2012-08-451864

167. Okada, S. *et al.* Enrichment and characterization of murine hematopoietic stem cells that express c-kit molecule. *Blood* 78, 1706–1712 (1991).

168. Li, C. L. & Johnson, G. R. Murine hematopoietic stem and progenitor cells: I. Enrichment and biologic characterization. *Blood* 85, 1472–1479 (1995).

169. Orlic, D., Fischer, R., Nishikawa, S., Nienhuis, A. W. & Bodine, D. M. Purification and characterization of heterogeneous pluripotent hematopoietic stem cell populations expressing high levels of c-kit receptor. *Blood* 82, 762–770 (1993).

170. Ikuta, K. & Weissman, I. L. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc. Natl. Acad. Sci. U.S.A.* 89, 1502–1506 (1992).

171. Magee, J. A. *et al.* Temporal Changes in PTEN and mTORC2 Regulation of Hematopoietic Stem Cell Self-Renewal and Leukemia Suppression. *Cell Stem Cell* (2012).

172. Winkler, I. G. *et al.* Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nature Medicine* (2012). doi:10.1038/nm.2969

173. Scheiermann, C. *et al.* Adrenergic nerves govern circadian leukocyte recruitment to tissues. *Immunity* 37, 290–301 (2012).

174. Kimura, Y. *et al.* c-Kit-mediated functional positioning of stem cells to their niches is essential for maintenance and regeneration of adult hematopoiesis. *PLoS ONE* 6, e26918 (2011).

175. Minot, C. S. On a hitherto unrecognized form of blood circulation without capillaries in the organs of vertebrates. *J Boston Soc Med Sci* 4, 133–134 (1900).

176. Doan, C. A. *Contributions to Embryology*. XIV, 27–45 (Carnegie Institution of Washington, 1922).

177. Pease, D. C. An electron microscopic study of red bone marrow. *Blood* 11, 501–526 (1956).

178. Hooper, A. T. *et al.* Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. *Cell Stem Cell* 4, 263–274 (2009).

179. Butler, J. M., Kobayashi, H. & Rafii, S. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nature Publishing Group* 10, 138–146 (2010).

180. Kobayashi, H. *et al.* Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nature Cell Biology* 12, 1046–1056 (2010).

181. Nilsson, S. K. *et al.* Hyaluronan is synthesized by primitive hemopoietic cells, participates in their lodgment at the endosteum following transplantation, and is involved in the regulation of their proliferation and differentiation in vitro. *Blood* 101, 856–862 (2003).

182. Avigdor, A. *et al.* CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow. *Blood* 103, 2981–2989 (2004).

183. Goncharova, V. *et al.* Hyaluronan expressed by the hematopoietic microenvironment is required for bone marrow hematopoiesis. *J. Biol. Chem.* 287, 25419–25433 (2012).

184. Himburg, H. A. *et al.* Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. *Nature Medicine* 16, 475–482 (2010).

185. Himburg, H. A. *et al.* Pleiotrophin Regulates the Retention and Self-Renewal of Hematopoietic Stem Cells in the Bone Marrow Vascular Niche. *CellReports* (2012). doi:10.1016/j.celrep.2012.09.002

186. Sipkins, D. A. *et al.* In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. *Nature Cell Biology* 435, 969–973 (2005).

187. Katayama, Y. *et al.* PSGL-1 participates in E-selectin-mediated progenitor homing to bone marrow: evidence for cooperation between E-selectin ligands and alpha4 integrin. *Blood* 102, 2060–2067 (2003).

188. Imai, K. *et al.* Selective secretion of chemoattractants for haemopoietic progenitor cells by bone marrow endothelial cells: a possible role in homing of haemopoietic progenitor cells to bone marrow. *Br. J. Haematol.* 106, 905–911 (1999).

189. Lane, W. J. *et al.* Stromal-derived factor 1-induced megakaryocyte migration and platelet production is dependent on matrix metalloproteinases. *Blood* 96, 4152–4159 (2000).

190. Dar, A. *et al.* Chemokine receptor CXCR4-dependent internalization and resecretion of functional chemokine SDF-1 by bone marrow endothelial and stromal cells. *Nat. Immunol.* 6, 1038–1046 (2005).

191. Jacobsen, K., Kravitz, J., Kincade, P. W. & Osmond, D. G. Adhesion receptors on bone marrow stromal cells: in vivo expression of vascular cell adhesion molecule-1 by reticular cells and sinusoidal endothelium

in normal and gamma-irradiated mice. *Blood* 87, 73–82 (1996).

192. Frenette, P. S., Subbarao, S., Mazo, I. B., Andrian, von, U. H. & Wagner, D. D. Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow. *Proc. Natl. Acad. Sci. U.S.A.* 95, 14423–14428 (1998).

193. Papayannopoulou, T., Priestley, G. V., Nakamoto, B., Zafiropoulos, V. & Scott, L. M. Molecular pathways in bone marrow homing: dominant role of alpha(4)beta(1) over beta(2)-integrins and selectins. *Blood* 98, 2403–2411 (2001).

194. Matrosova, V. Y., Orlovskaya, I. A., Serobyan, N. & Khaldoyanidi, S. K. Hyaluronic acid facilitates the recovery of hematopoiesis following 5-fluorouracil administration. *Stem Cells* 22, 544–555 (2004).

195. Kopp, H.-G. *et al.* Thrombospondins deployed by thrombopoietic cells determine angiogenic switch and extent of revascularization. *J. Clin. Invest.* 116, 3277–3291 (2006).

196. Carmeliet, P. & Jain, R. K. Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473, 298–307 (2011).

197. Lennon, F. E. & Singleton, P. A. Hyaluronan regulation of vascular integrity. *Am J Cardiovasc Dis* 1, 200–213 (2011).

198. Reya, T., Morrison, S. J., Clarke, M. F. & Weissman, I. L. Stem cells, cancer, and cancer stem cells. *Nature* 414, 105–111 (2001).

199. Bhardwaj, G. *et al.* Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat. Immunol.* 2, 172–180 (2001).

200. Trowbridge, J. J., Scott, M. P. & Bhatia, M. Hedgehog modulates cell cycle regulators in stem cells to control hematopoietic regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 103, 14134–14139 (2006).

201. Varnum-Finney, B. *et al.* The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. *Blood* 91, 4084–4091 (1998).

202. Varnum-Finney, B. *et al.* Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nature Medicine* 6, 1278–1281 (2000).

203. Karanu, F. N. *et al.* Human homologues of Delta-1 and Delta-4 function as mitogenic regulators of primitive human hematopoietic cells. *Blood* 97, 1960–1967 (2001).

204. Varnum-Finney, B., Brashem-Stein, C. & Bernstein, I. D. Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. *Blood* 101, 1784–1789 (2003).

205. Kunisato, A. *et al.* HES-1 preserves purified hematopoietic stem cells ex vivo and accumulates side population cells in vivo. *Blood* 101, 1777–1783 (2003).

206. Stier, S., Cheng, T., Dombkowski, D., Carlesso, N. & Scadden, D. T. Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. *Blood* 99, 2369–2378 (2002).

207. Duncan, A. W. *et al.* Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat. Immunol.* 6, 314–322 (2005).

208. Reya, T. *et al.* A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature* 423, 409–414 (2003).

209. Fleming, H. E. *et al.* Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. *Cell Stem Cell* 2, 274–283 (2008).

210. Willert, K. *et al.* Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 423, 448–452 (2003).

211. Luis, T. C. *et al.* Wnt3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation. *Blood* 113, 546–554 (2009).

212. Hofmann, I. *et al.* Hedgehog signaling is dispensable for adult murine hematopoietic stem cell function and hematopoiesis. *Cell Stem Cell* 4, 559–567 (2009).

213. Gao, J. *et al.* Hedgehog signaling is dispensable for adult hematopoietic stem cell function. *Cell Stem Cell*

4, 548–558 (2009).

214. Mancini, S. J. C. *et al.* Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood* 105, 2340–2342 (2005).

215. Maillard, I. *et al.* Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells. *Cell Stem Cell* 2, 356–366 (2008).

216. Malhotra, S. & Kincade, P. W. Wnt-related molecules and signaling pathway equilibrium in hematopoiesis. *Cell Stem Cell* 4, 27–36 (2009).

217. Gustafsson, M. V. *et al.* Hypoxia Requires Notch Signaling to Maintain the Undifferentiated Cell State. *Developmental Cell* 9, 617–628 (2005).

218. Naka, K., Muraguchi, T., Hoshii, T. & Hirao, A. Regulation of reactive oxygen species and genomic stability in hematopoietic stem cells. *Antioxid. Redox Signal.* 10, 1883–1894 (2008).

219. Ito, K. *et al.* Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nature Medicine* 12, 446–451 (2006).

220. Jang, Y.-Y. & Sharkis, S. J. A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. *Blood* 110, 3056–3063 (2007).

221. Yahata, T. *et al.* Accumulation of oxidative DNA damage restricts the self-renewal capacity of human hematopoietic stem cells. *Blood* (2011). doi:10.1182/blood-2011-01-330050

222. Artavanis-Tsakonas, S., Rand, M. D. & Lake, R. J. Notch signaling: cell fate control and signal integration in development. *Science* 284, 770–776 (1999).

223. Tien, A.-C., Rajan, A. & Bellen, H. J. A Notch updated. *The Journal of Cell Biology* 184, 621–629 (2009).

224. Sandy, A. R. & Maillard, I. Notch signaling in the hematopoietic system. *Expert Opin Biol Ther* 9, 1383–1398 (2009).

225. Kopan, R. & Ilagan, M. X. G. The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism. *Cell* 137, 216–233 (2009).

226. Iso, T., Kedes, L. & Hamamori, Y. HES and HERP families: multiple effectors of the Notch signaling pathway. *J. Cell. Physiol.* 194, 237–255 (2003).

227. Cabrera, C. V. Lateral inhibition and cell fate during neurogenesis in Drosophila: the interactions between scute, Notch and Delta. *Development* 110, 733–742 (1990).

228. Itoh, M. *et al.* Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. *Developmental Cell* 4, 67–82 (2003).

229. Hobson, B. & Denekamp, J. Endothelial proliferation in tumours and normal tissues: continuous labelling studies. *Br. J. Cancer* 49, 405–413 (1984).

230. Carmeliet, P. Angiogenesis in health and disease. *Nature Medicine* 9, 653–660 (2003).

231. Ferrara, N. & Kerbel, R. S. Angiogenesis as a therapeutic target. *Nature* 438, 967–974 (2005).

232. Tang, N. *et al.* Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. *Cancer Cell* 6, 485–495 (2004).

233. Diez, H. *et al.* Hypoxia-mediated activation of Dll4-Notch-Hey2 signaling in endothelial progenitor cells and adoption of arterial cell fate. *Experimental Cell Research* 313, 1–9 (2007).

234. Gerhardt, H. *et al.* VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *The Journal of Cell Biology* 161, 1163–1177 (2003).

235. van Hinsbergh, V. W. M. & Koolwijk, P. Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead. *Cardiovasc. Res.* 78, 203–212 (2008).

236. Phng, L. K. & Gerhardt, H. Angiogenesis: a team effort coordinated by notch. *Developmental Cell* 16, 196–208 (2009).

237. Adams, R. H. & Alitalo, K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol* 8, 464–478 (2007).

238. Estrach, S. *et al.* Laminin-binding integrins induce Dll4 expression and Notch signaling in endothelial cells. *Circ. Res.* 109, 172–182 (2011).

239. Duarte, A. *et al.* Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes & Development* 18, 2474–2478 (2004).

240. Claxton, S. & Fruttiger, M. Periodic Delta-like 4 expression in developing retinal arteries. *Gene Expr. Patterns* 5, 123–127 (2004).

241. Gale, N. W. *et al.* Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15949–15954 (2004).

242. Hellström, M. *et al.* Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* 445, 776–780 (2007).

243. Lobov, I. B. *et al.* Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3219–3224 (2007).

244. Shutter, J. R. *et al.* Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes & Development* 14, 1313–1318 (2000).

245. Tammela, T. *et al.* Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature* 454, 656–660 (2008).

246. Tammela, T. *et al.* VEGFR-3 controls tip to stalk conversion at vessel fusion sites by reinforcing Notch signalling. *Nature Cell Biology* 13, 1202–1213 (2011).

247. Benedito, R. *et al.* Notch-dependent VEGFR3 upregulation allows angiogenesis without VEGF-VEGFR2 signalling. *Nature* 484, 110–114 (2012).

248. Benedito, R. *et al.* The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* 137, 1124–1135 (2009).

249. Rao, P. K. *et al.* Isolation and characterization of the notch ligand delta4. *Experimental Cell Research* 260, 379–386 (2000).

250. Thurston, G., Noguera-Troise, I. & Yancopoulos, G. D. The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth. *Nat. Rev. Cancer* 7, 327–331 (2007).

251. Krebs, L. T. *et al.* Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes & Development* 18, 2469–2473 (2004).

252. Patel, N. S. *et al.* Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. *Cancer Research* 65, 8690–8697 (2005).

253. Patel, N. S. *et al.* Up-regulation of endothelial delta-like 4 expression correlates with vessel maturation in bladder cancer. *Clin. Cancer Res.* 12, 4836–4844 (2006).

254. Indraccolo, S. *et al.* Cross-talk between Tumor and Endothelial Cells Involving the Notch3-Dll4 Interaction Marks Escape from Tumor Dormancy. *Cancer Research* 69, 1314–1323 (2009).

255. Noguera-Troise, I. *et al.* Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* 444, 1032–1037 (2006).

256. Ridgway, J. *et al.* Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature* 444, 1083–1087 (2006).

257. Scehnet, J. S. *et al.* Inhibition of Dll4-mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion. *Blood* 109, 4753–4760 (2007).

258. OncoMed Pharmaceuticals, Inc. A Phase 1 Dose Escalation Study of OMP-21M18 in Subjects With Solid Tumors. In: ClinicalTrials.gov. *Bethesda (MD): National Library of Medicine (US)* 1–3 (2013). at <http://clinicaltrials.gov/ct2/show/study/NCT00744562>

259. Regeneron PhamaceuticalsSanofi. A Multiple-Ascending-Dose Study of the Safety and Tolerability of REGN421(SAR153192) in Patients With Advanced Solid Malignancies. In: ClinicalTrials.gov. *Bethesda (MD): National Library of Medicine (US)* 1–3 (2013). at <http://clinicaltrials.gov/ct2/show/study/NCT00871559>

260. Suchting, S. *et al.* The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3225–3230 (2007).

261. Lobov, I. B. *et al.* The Dll4/Notch pathway controls postangiogenic blood vessel remodeling and regres-

sion by modulating vasoconstriction and blood flow. *Blood* 117, 6728–6737 (2011).

262. Dando, J. S. *et al.* Notch/Delta4 Interaction in Human Embryonic Liver CD34 +CD38 –Cells: Positive Influence on BFU-E Production and LTC-IC Potential Maintenance. *Stem Cells* 23, 550–560 (2005).

263. Lahmar, M. *et al.* Distinct effects of the soluble versus membrane-bound forms of the notch ligand delta-4 on human CD34+CD38low cell expansion and differentiation. *Stem Cells* 26, 621–629 (2008).

264. Lauret, E. *et al.* Membrane-bound delta-4 notch ligand reduces the proliferative activity of primitive human hematopoietic CD34+CD38low cells while maintaining their LTC-IC potential. *Leukemia* 18, 788–797 (2004).

265. Dorsch, M. *et al.* Ectopic expression of Delta4 impairs hematopoietic development and leads to lymphoproliferative disease. *Blood* 100, 2046–2055 (2002).

266. Yan, X. Q. *et al.* A novel Notch ligand, Dll4, induces T-cell leukemia/lymphoma when overexpressed in mice by retroviral-mediated gene transfer. *Blood* 98, 3793–3799 (2001).

267. Poirault-Chassac, S. *et al.* Notch/Delta4 signaling inhibits human megakaryocytic terminal differentiation. *Blood* 116, 5670–5678 (2010).

268. Laranjeiro, R. *et al.* The Notch Ligand Delta-Like 4 Regulates Multiple Stages of Early Hemato-Vascular Development. *PLoS ONE* 7, e34553 (2012).

269. Lee, S.-U. *et al.* LRF-mediated Dll4 repression in erythroblasts is necessary for hematopoietic stem cell maintenance. *Blood* 121, 918–929 (2013).

270. Koch, U. *et al.* Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *Journal of Experimental Medicine* 205, 2515–2523 (2008).

271. Hozumi, K. *et al.* Delta-like 4 is indispensable in thymic environment specific for T cell development. *Journal of Experimental Medicine* 205, 2507–2513 (2008).

272. Mohtashami, M. *et al.* Direct comparison of Dll1- and Dll4-mediated Notch activation levels shows differential lymphomyeloid lineage commitment outcomes. *The Journal of Immunology* 185, 867–876 (2010).

273. Tsiftsoglou, A. S., Pappas, I. S. & Vizirianakis, I. S. Mechanisms involved in the induced differentiation of leukemia cells. *Pharmacol. Ther.* 100, 257–290 (2003).

274. Pui, C.-H. Acute lymphoblastic leukemia: introduction. *Semin. Hematol.* 46, 1–2 (2009).

275. Hanahan, D. & Weinberg, R. A. Hallmarks of Cancer: The Next Generation. *Cell* 144, 646–674 (2011).

276. Doan, P. L. & Chute, J. P. The vascular niche: home for normal and malignant hematopoietic stem cells. *Leukemia* 26, 54–62 (2012).

277. Chiorazzi, N. & Ferrarini, M. Cellular origin(s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities. *Blood* 117, 1781–1791 (2011).

278. Crews, L. A. & Jamieson, C. H. M. Selective elimination of leukemia stem cells: Hitting a moving target. *Cancer Lett.* (2012). doi:10.1016/j.canlet.2012.08.006

279. Ayala, F., Dewar, R., Kieran, M. & Kalluri, R. Contribution of bone microenvironment to leukemogenesis and leukemia progression. *Leukemia* 23, 2233–2241 (2009).

280. Colmone, A. *et al.* Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells. *Science* 322, 1861–1865 (2008).

281. Ellisen, L. W. *et al.* TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* 66, 649–661 (1991).

282. Weng, A. P. *et al.* Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* 306, 269–271 (2004).

283. Zhang, J. *et al.* The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. *Nature* 481, 157–163 (2012).

284. Lewis, H. D. *et al.* Apoptosis in T cell acute lymphoblastic leukemia cells after cell cycle arrest induced by pharmacological inhibition of notch signaling. *Chem. Biol.* 14, 209–219 (2007).

285. Kogoshi, H., Sato, T., Koyama, T., Nara, N. & Tohda, S. Gamma-secretase inhibitors suppress the growth

of leukemia and lymphoma cells. *Oncol. Rep.* 18, 77–80 (2007).

286. Li, G.-H. *et al.* Notch signaling maintains proliferation and survival of the HL60 human promyelocytic leukemia cell line and promotes the phosphorylation of the Rb protein. *Mol. Cell. Biochem.* 340, 7–14 (2010).

287. Meng, X. *et al.* GSI-I (Z-LLNle-CHO) inhibits γ-secretase and the proteosome to trigger cell death in precursor-B acute lymphoblastic leukemia. *Leukemia* 25, 1135–1146 (2011).

288. Tohda, S. *et al.* Establishment of a novel B-cell lymphoma cell line with suppressed growth by gamma-secretase inhibitors. *Leuk. Res.* 30, 1385–1390 (2006).

289. Pear, W. S. *et al.* Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J. Exp. Med.* 183, 2283–2291 (1996).

290. Bellavia, D. *et al.* Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice. *EMBO J.* 19, 3337–3348 (2000).

291. Indraccolo, S., Minuzzo, S., Masiero, M. & Amadori, A. Ligand-driven activation of the notch pathway in T-ALL and solid tumors: why Not(ch)? *Cell Cycle* 9, 80–85 (2010).

292. Real, C. *et al.* Bone marrow-derived endothelial progenitors expressing Delta-like 4 (Dll4) regulate tumor angiogenesis. *PLoS ONE* 6, e18323 (2011).

293. Masiero, M. *et al.* Notch3-mediated regulation of MKP-1 levels promotes survival of T acute lymphoblastic leukemia cells. *Leukemia* 25, 588–598 (2011).

294. Zhang, J. *et al.* Prognostic impact of δ-like ligand 4 and Notch1 in acute myeloid leukemia. *Oncol. Rep.* 28, 1503–1511 (2012).

295. Zhang, J. *et al.* Cross-talk between Leukemic and Endothelial cells Promotes Angiogenesis by VEGF Activation of the Notch/Dll4 Pathway. *Carcinogenesis* (2012). doi:10.1093/carcin/bgs386

296. Sarmento, L. M. *et al.* Notch1 modulates timing of G1-S progression by inducing SKP2 transcription and p27 Kip1 degradation. *J. Exp. Med.* 202, 157–168 (2005).

2

CONTEXT- AND CELL-DEPENDENT EFFECTS OF DELTA-LIKE 4 TARGETING IN THE BONE MARROW MICROENVIRONMENT

Results published in Remédio, L., Carvalho, T., Caiado, F., Bastos-Carvalho, A., Martins, D., Duarte, A., Yagita, H., and Dias, S. (2012). Context- and cell-dependent effects of Delta-like 4 targeting in the bone marrow microenvironment. PLoS ONE 7 (12), e52450.

CONTENTS

ABSTRACT **50**

INTRODUCTION **51**

METHODS **53**

Animals and experimental design 53

Sample collection 54

Bone Marrow Transplants 54

Cell culture 54

In vitro colony forming assays 55

Flow cytometry 55

Histological and immunohistochemical analysis 57

Vascular perfusion 57

Western blotting 58

Reverse transcriptase PCR (RT–PCR) 58

Statistical analysis 59

RESULTS **62**

Systemic anti-Dll4 treatment interferes with the BM vascular niche 62

Specific effects of anti-Dll4 treatment on endothelial cells 67

Anti-Dll4 treatment perturbs hematopoietic recovery following irradiation 71

Anti-Dll4 treatment of donor BM improves hematopoietic recovery following transplantation into lethally irradiated recipients 74

DISCUSSION **75**

REFERENCES **78**

ABSTRACT

Delta-like 4 (Dll4) is a ligand of the Notch pathway family which has been widely studied in the context of tumor angiogenesis, its blockade shown to result in non-productive angiogenesis and halted tumor growth. As Dll4 inhibitors enter the clinic, there is an emerging need to understand its side effects, namely the systemic consequences of Dll4:Notch blockade in tissues other than tumors. The present study focused on the effects of systemic anti-Dll4 targeting in the bone marrow (BM) microenvironment. Here we show that Dll4 blockade with monoclonal antibodies perturbs the BM vascular niche of sub-lethally irradiated mice, resulting in increased $CD31^+$, VE-$Cadherin^+$ and $c\text{-}kit^+$ vessel density, and also increased megakaryocytes, whereas $CD105^+$, $VEGFR3^+$, SMA^+ and $lectin^+$ vessel density remained unaltered. We investigated also the expression of angiocrine genes upon Dll4 treatment *in vivo*, and demonstrate that IGFbp2, IGFbp3, Angpt2, Dll4, DHH and VEGF-A are upregulated, while FGF1 and CSF2 are reduced. In *vitro* treatment of endothelial cells with anti-Dll4 reduced Akt phosphorylation while maintaining similar levels of Erk 1/2 phosphorylation. Besides its effects in the BM vascular niche, anti-Dll4 treatment perturbed hematopoiesis, as evidenced by increased myeloid ($CD11b^+$), decreased B ($B220^+$) and T ($CD3^+$) lymphoid BM content of treated mice, with a corresponding increase in myeloid circulating cells. Moreover, anti-Dll4 treatment also increased the number of CFU-M and -G colonies in methylcellulose assays, independently of Notch1. Finally, anti-Dll4 treatment of donor BM improved the hematopoietic recovery of lethally irradiated recipients in a transplant setting. Together, our data reveals the hematopoietic (BM) effects of systemic anti-Dll4 treatment result from qualitative vascular changes and also direct hematopoietic cell modulation, which may be favorable in a transplant setting.

INTRODUCTION

Hematopoiesis is the process by which new blood cells are generated and occurs mainly in the adult bone marrow (BM). The importance of the microenvironment of the BM in hematopoiesis has been clarified with the proposal of "stem cell niches", in which the endosteal and vascular niches would support hematopoietic stem cells (HSCs) self-renewal, proliferation, and differentiation [1-4]. However, recent findings have suggested this interpretation of the BM stem cell niches may be too simplistic [5,6]. Interestingly, the vascular niche is not only critical for HSC maintenance [7-9] and differentiation [10], but also for hematopoietic reconstitution and recovery [11-15]. Mechanistically, the BM endothelial cells (ECs) were shown to express different "angiocrine" genes, whose production is dependent on the activation of Akt or p42/44 mitogen-activated protein kinase (MAPK) signaling pathways [16], and whose function is to restore hematopoiesis following insults such as irradiation. Therefore, targeting the BM vascular niche and "angiocrine" genes production to modulate hematopoietic recovery and function may be of clinical relevance. We found Delta-like 4 (Dll4, a ligand of the Notch signaling pathway expressed by BM ECs) targeting to potentially fulfill this aim.

Blockade of Dll4-mediated Notch signaling has been described as a modulator of angiogenesis. Indeed, its inhibition, by promoting non-productive angiogenesis, was shown to be an effective treatment strategy in pre-clinical solid tumor models [17-20], and is already being tested in clinical trials [21,22].

We have explored the effects of Dll4 blockade in the BM vascular niche using two strategies, first by using different EC markers, to assess qualitative changes in BM vasculature, and secondly by exploring the modulation of "angiocrine" genes *in vivo* and EC-specific activation of signaling pathways *in vitro*. To characterize the phenotypic response of the BM vascular niche to anti-Dll4 antibody treatment, we used different

EC markers (CD31, CD105, vascular endothelial (VE)-Cadherin, vascular endothelial growth factor receptor 3 (VEGFR3) and *Lycopersicon esculentum* lectin [23-25]), a pericyte marker (smooth muscle actin, SMA [26]) and by counting megakaryocyte numbers (which are part of the BM vascular niche, and are $CD41^+$ [27-29]). Additionally, we assessed the effect of Dll4 blockade in modulating the expression of "angiocrine" genes [16] and activation of signaling pathways on BM ECs *in vitro*.

We also determined how Dll4 systemic blockade interfered with hematopoiesis by directly affecting hematopoietic cells. Dll4 has been shown to be involved in HSCs self-renewal and proliferation [30-32], megakaryocytic differentiation [33,34] and lymphoid modulation [33,35-37]. However, the hematopoietic effects of Dll4 blockade, namely in the setting of perturbed BM function, have not been previously shown.

We have performed *in vivo* phenotypic characterization of the main BM hematopoietic lineages following anti-Dll4 treatment, *in vitro* functional assays to identify hematopoietic cell-specific modulation of anti-Dll4, and an *in vivo* BM transplant (BMT) following lethal irradiation. For the *in vivo* characterization of the main BM hematopoietic lineages we quantified myeloid ($CD11b^+$) and lymphoid (B, $B220^+$ and T, $CD3^+$) BM content [38-41]. Additionally, we measured hematopoietic stem/progenitor cells (HSPCs; stem cell antigen (Sca)-1^+ fetal liver kinase (Flk)-1^- [42,43]) and endothelial progenitor cells (EPCs; $Sca1^+Flk1^+$ [44-46]), in BM and peripheral blood (PB). The effects of anti-Dll4 treatment in HSPCs commitment and differentiation was assessed *in vitro* by performing colony-forming units (CFU) assays in methylcellulose [47,48].

We show that systemic Dll4 blockade affects the BM vascular niche and hematopoietic cell differentiation, while having limited effects on the expression of "angiocrine" genes or on EC activation. Interestingly, in a BMT setting, anti-Dll4 treatment of donor mice results in a faster lymphoid and erythroid recovery of recipient mice.

Together, we show that anti-Dll4 treatment perturbs BM recovery following irradiation, which can be clinically relevant in a BMT setting.

METHODS

Animals and experimental design

The following animal experiments were performed with the approval of the Instituto Gulbenkian de Ciência Animal Care Committee and Review Board.

Balb/c mice (6-8 weeks old) were sub-lethally irradiated (300rad), and subjected to treatment with neutralizing anti-mouse Dll4 antibody (HMD4-2) [20,49,50], 12.5 g/kg, intraperitoneally (IP), every 2 days or every 3 days, for 15 days, starting 1 day after irradiation. In parallel, control mice were injected with phosphate-buffered saline (PBS). All experiments refer to 15-20 days counting from the day of irradiation. Each irradiated group consisted of 3 control and 3 anti-Dll4 treated animals, and the experiments were performed 3 times.

The Dll4 knockout mice experiments were performed with the approval of the Faculty of Veterinary Medicine of Lisbon Ethics and Animal Welfare Committee. Dll4 conditional knockout mice ($Dll4^{lox/lox}$) were generated as follows. Conditional KO Dll4 vector with two loxP sequences flanking the tree first gene exons was inserted in EE cells by electroporation. The neomicine resistant clones were selected, injected in blastocysts and transferred to pseudo-pregnant females. The offspring were crossed with h-ActB-flp mice to remove neoR, and the resultant littermates were crossed to obtain $Flp^{-/-}$. These mice were then crossed with $VECad^{CreERT2}$ mice, a gift from Dr. Ralph Adams, to produce a tamoxifen-inducible endothelial-specific Dll4 loss-of-function line ($VECad^{CreERT2}Dll4^{lox/lox}$). Tamoxifen induction was performed for 5 days, 50mg/kg/day. All experiments refer to 31-34 days counting from the first day of induction. Each group consisted of 12 $Dll4^{lox/lox}$ and 11

$VECad^{CreERT2}Dll4^{lox/lox}$ animals.

Sample collection

Peripheral blood was collected from the heart in EDTA-coated tubes (Multivette 600, Sarstedt, Nümbrecht, Germany) and centrifuged at 1200 rpm for 5 minutes.

BM was flushed from the long bones with PBS 0.5% BSA and centrifuged at 800 rpm for 15 minutes. PB and BM cells were collected for FACS analysis.

Femur BM was flushed with PBS and immediately centrifuged at 800 rpm for 15 minutes. Plasma was then collected for enzyme-linked immunosorbent assay (ELISA) analysis.

Bone Marrow Transplants

Balb/c mice (6 weeks old) were lethally irradiated (800rad), and subjected to BMT 24 hours later. Cells for BMT were collected from the femur of previously treated or control animals (two recipients per donor animal), on day 15 of treatment. Viable nucleated cells were counted in a Countless Automated Cell Counter (Invitrogen, Carlsbad, CA). $2.5x10^6$ total BM cells were injected intravenously. BM for BMT was collected from 3 control and 3 anti-Dll4 treated animals. Recipient animals were treated with enrofloxacin 10mg/kg every day for 7 days post-irradiation.

Complete blood counts (CBC) of tail vein PB was performed at weeks 1 and 2 post-transplantation.

Cell culture

Human umbilical cord vein ECs (HUVECs) (Clonetics, Lonza, Switzerland) were cultured in EBM-2 supplemented with EGM-2 Single-

Quots, 2mg/mL BBE (Lonza, Walkersville, MD) and 10% heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen, Carlsbad, CA).

Murine bone marrow-derived stromal cell line S17 was cultured in complete medium – Roswell Park Memorial Institute (RPMI) 1640 medium, 2mM L-Glutamine, antibiotic-antimycotic (all from Gibco Invitrogen, Carlsbad, CA) and 50μM β-mercaptoethanol (Sigma-Aldrich, Germany) – plus 10% FBS.

In vitro colony forming assays

Bone marrow mononuclear Lineage $(Lin)^{-}Sca1^{+}$ cells (10^4), collected from anti-Dll4 treated and control animals and sorted in FacsAria (Becton Dickinson, Franklin Lakes, NJ), were plated onto cytokine-supplemented methylcellulose medium (MethoCult GF M3434, Stem Cell Technologies, Vancouver, BC, Canada). Resulting colonies are single-cell derived and represent the original cell's identity [47,48]. Colony type was scored after 1 and 2 weeks of culture, according to the manufacturer's instructions.

Human cord blood mononuclear cells were lineage depleted using lineage cell depletion kit, as shown in Table 2.1. 10^4 Lin^{-} cells were plated onto cytokine-supplemented methylcellulose medium (MethoCult GF H4434, Stem Cell Technologies, Vancouver, BC, Canada). Treatment with neutralizing anti-human Dll4 antibody (MHD4-46) [51,52], 50 μg/mL, and/or anti-human Notch1 antibody (MHN1-128) [53], 10 μg/mL, started the day after the establishment of the culture and was performed every 2 days. Colony type was scored after 1 week of culture, according to the manufacturer's instructions.

Flow cytometry

Bone marrow and PB mononuclear cells were stained for T, B, myeloid and progenitor cell markers, using the antibodies indicated on Table

2.1, 1 h at 4ºC. Bone marrow cells were stained for megakaryocytes, following the same protocol.

Flow cytometry was performed on FACSCalibur and analyzed with Cell Quest Software (Becton Dickinson, Franklin Lakes, NJ).

Table 2.1. Antibodies list.

Antigen	Application	Dilution	Clone	Brand
β-actin	Western Blotting	1:5000	AC-15	SIGMA A5441
Akt	Western Blotting	1:1000	11E7	CellSignaling #4685
B220-PE	Flow cytometry	1:100	RA3 6B2	Produced in Instituto Gulbenkian de Ciência
CD3-PE	Flow cytometry	1:100	145.2C11	Produced in Instituto Gulbenkian de Ciência
CD11b-FITC	Flow cytometry	1:100	M1/70	BD 553310
CD31	Immunohistochemistry, HIAR	1:150	Polyclonal	SIGMA SAB4502167
CD41	Flow cytometry	1:100	MWReg30	BD 553848
CD105 (Endoglin)	Immunohistochemistry and fluorescence, HIAR	1:150	Polyclonal	R&D AF1320
c-kit (CD117)	Immunofluorescence, HIAR	1:200	Polyclonal	DAKO A4502
Erk1/2	Western Blotting	1:1000	137F5	CellSignaling #4695
Flk1-PE	Flow cytometry	1:100	AVAS 12α1	BD555308
SMA	Immunohistochemistry, PIER	1:500	HHF35	DAKO HHF35
Lineage-Biotin	Flow cytometry (sorter)	According to manufacturer's instructions		Miltenyi Biotec 130-090-858
Lineage cell depletion kit, human	Cell isolation	According to manufacturer's instructions		Miltenyi Biotec 130-092-211
pAkt (Ser473)	Western Blotting	1:1000	D9E	CellSignaling #4060
pErk1/2 (Thr202/Tyr204)	Western Blotting	1:2000	D13.14.4E	CellSignaling #4370
Sca1-FITC	Flow cytometry	1:100	E13-161.7	BD 553335
Sca1-FITC	Flow cytometry (sorter)	According to manufacturer's instructions		Miltenyi Biotec 130-092-529
TO-PRO 3	Immunofluorescence	1:1000		Invitrogen T3605
VE-Cadherin	Immunohistochemistry, PIER	1:150	Polyclonal	R&D AF1002
VEGFR3	Immunofluorescence, HIAR	1:50	AFL4	BD 552857

Antigen	Application	Dilution	Clone	Brand
Streptavidin-APC	Flow cytometry (sorter)	1:100		BD 554067
Anti-Goat, peroxidase	Immunohistochemistry	ready-to-use		VectorLabs MP-7405
Anti-Mouse, peroxidase	Immunohistochemistry	ready-to-use		DAKO K4007
Anti-Rabbit, peroxidase	Immunohistochemistry	ready-to-use		DAKO K4011
Anti-Rat, peroxidase	Immunohistochemistry	ready-to-use		VectorLabs MP-7444
Anti-Goat FITC	Immunofluorescence	1:100	Polyclonal	Jackson 705-095-147
Anti-Rabbit-594	Immunofluorescence	1:500	Polyclonal	Invitrogen A21207
Anti-Rabbit, HRP	Western Blotting	1:5000		ThermoScientific
Anti-Mouse, HRP	Western Blotting	1:5000		ThermoScientific

Histological and immunohistochemical analysis

Livers were formalin-fixed and processed for routine histopathology and immunohistochemistry. Bones were formalin-fixed, EDTA-decalcified and processed for routine histopathology. Immunohistochemistry for the antigens indicated on Table 2.1 was performed in the humerus, on 3μm slices, at 3 distinct levels for each bone/mouse (40μm distance). Sections were incubated with primary antibody at room temperature for 1h, immunostaining proceeded according to the visualization system manufacturer's instructions and counterstained with Mayer's hematoxylin.

Immunofluorescence for the antigens indicated on Table 2.1 was performed in the humerus, on 3μm slices. Primary antibodies were incubated at room temperature for 1 hour, secondary antibodies were incubated at room temperature for 2 hours. Slides were mounted with Vectashield mounting medium with DAPI (VectorLaboratories, Burlingame, CA).

Vascular perfusion

Fluorescein isothiocyanate (FITC) Lycopersicon esculentum lectin (VectorLaboratories, Burlingame, CA) was injected in the tail vein (100µg, from a 500µg/mL solution). Mice were euthanized 5 minutes later, and perfused with 4% paraformaldehyde (PFA) in PBS. Femur BM was then flushed off and further fixed in 4% PFA overnight, dehydrated in a sucrose gradient for one day, and cryopreserved in Tissue-Tek Optimum Cutting Temperature (Sakura, Torrance, CA).

Cryosections (15µm) were stained with ToPro-3 (Table 2.1) plus 100µg/mL ribonuclease A (Sigma-Aldrich, Germany) at 4ºC overnight, to visualize nuclei, and mounted in Mowiol 4-88 (pH 8.5 in Tris-HCl and glycerol; Calbiochem Merk Millipore, Darmstadt, Germany).

Western blotting

Third passage HUVEC at 70% confluence were starved with EBM-2 plus 1% FBS for 17 hours, and treated with neutralizing anti-human Dll4 antibody (MHD4-46) [51,52], 50 µg/mL, or PBS, for 2 hours. Cells were then lysed with RIPA buffer (20mM Tris pH 7.5, 150mM NaCl, 5mM KCl, 5mM MgCl, 1% Triton X-100, protease inhibitor cocktail and 1mM sodium orthovanadate), and equal amounts of proteins were subjected to SDS–polyacrylamide gel electrophoresis with 12% Mini-Protean TGX precast gel (BioRad, US). Proteins were transferred onto nitrocellulose membrane (Hybond-C Extra, GE Healthcare Life Sciences, Roosendaal, Netherlands) and subjected to standard immunoblotting with the antibodies indicated on Table 2.1.

Reverse transcriptase PCR (RT–PCR)

For *in vivo* assessments, total BM from control or anti-Dll4 treated mice was flushed off in PBS, centrifuged 1200rpm 5min, and collected to TRIzol Reagent (Invitrogen, Carlsbad, CA). For *in vitro* assessments,

third passage HUVEC at 70% confluence were starved with EBM-2 plus 1% FBS overnight, and treated with neutralizing anti-human Dll4 antibody (MHD4-46) [51,52], 50 μg/mL, or PBS, for 16 hours, then collected to TRIzol Reagent (Invitrogen, Carlsbad, CA).

RNA was extracted according to the manufacturer's instructions. cDNA was produced with SuperScript II (Invitrogen, Carlsbad, CA) by using random-sequence hexamer primers (Roche Applied Science, Indianapolis, IN). Real-time PCR was performed with Power SYBR Green PCR Master Mix in 7900HT Fast Real-Time PCR System (both from Applied Biosystems, Foster City, CA). Amplification of 18S rRNA, hypoxanthine-guanine phosphoribosyl transferase (HPRT) and b2-microglobulin (β2MG) were used for sample normalization; data were analyzed using all these endogenous controls and plotted using HPRT only. Primer sequences are as described on Table 2.2.

RT-PCR data were analyzed by DataAssist software (Applied Biosystems Foster City, CA) using 18S, β2MG and HPRT as endogenous controls, and plotted using HPRT as endogenous control.

Statistical analysis

Results are expressed as mean ± standard error. Data were analyzed using unpaired two-tailed student's t test. P values of <0.05 were considered statistically significant.

Table 2.2. Primers list.

Mouse Primers		
m18S	F	CGCAGCTAGGAATAATGGAATAGG
	R	GCCTCAGTTCCGAAAACCAA
m 2MG	F	TCACGCCACCCACCGGAGAA
	R	TGTGAGGCGGGTGGAACTGTG
mHPRT	F	CAGCCCCAAAATGGTTAAGGTTGC
	R	CTGGCCTGTATCCAACACTTCGAG
mAngpt1	F	GCCTTTGCACTAAAGAAGGTGT
	R	CTGCACAGTCTCGAAATGGTT
mAngpt2	F	CAGCAGCACAAACTCGGAAAC
	R	TCGAGTCTTGTCGTCTGGTTTAG
mBMP4	F	GAGCCAACACTGTGAGGAGTTT
	R	CCCTGGGATGTTCTCCAGAT
mCD31	F	CGAGAACTTTGTGCTCATGGAA
	R	CTTGGCAGCGAAACACTAACAC
mCSF1	F	GCCACATGATTGGGAATGG
	R	AAAGGCAATCTGGCATGAAGTC
mCSF2	F	AGGGCGCCTTGAACATGA
	R	CACAGTCCGTTTCCGGAGTT
mCSF3	F	GCAGGCTCTATCGGGTATTTCC
	R	GCAACATCCAGCTGAAGCAA
mCXCR4	F	GGGACATCAGTCAGGGGGAT
	R	CTATCGGGGTAAAGGCGGTC
mDHH	F	CTACTACGAGTCCCGCAACC
	R	ACCGCCAGTGAGTTATCAGC
mDKK1	F	CTATGAGGGCGGGAACAAGT
	R	ATCTTCAGCGCAAGGGTAGG
mDll1	F	CGATTCCCCTTCGGCTTCAC
	R	GGGTTTTCTGTTGCGAGGTC
mDll4	F	TTTGTGACCAAGATCTCAACTACTGTAC
	R	CTTTGGCCCACTGTTGGAA
mFGF1	F	AGGAGCGACCAGCACATTC
	R	ATATACACTTCGCCCGCACTT
mFGF2	F	CGACCCACACGTCAAACTAC
	R	GTTGGCACACACTCCCTTGA
mIGF1	F	AACAAGCCCACAGGCTATGG
	R	AAGCAACACTCATCCACAATGC
mIGFbp2	F	CTGCACATCCCCAACTGTGA
	R	CGCTGTCCGTTCAGAGACAT
mIGFbp3	F	GCAGGCAGCCTAAGCACCTA
	R	CCTCCTCGGACTCACTGATGTT
mIGFbp5	F	AACGAAAAGAGCTACGGCGA
	R	GGTCTCTTCAGCCATCTCGG
mIL-3	F	CCACCTCAGCCCGCATCTGG
	R	AACATTCCACGGTTCCACGGTT
mIL-6	F	CTGGGAAATCGTGGAAATGAGA
	R	GCAAGTGCATCATCGTTGTTCAT
mIL-11	F	CAGCTGACGGAGATCACAGTCT
	R	AAGCTGCAAAGATCCCAATGTC
mJagged1	F	CCAGTGTTTCGTGCGCCCCT
	R	GCTGGAGGCTGGAGGACCGA
mJagged2	F	TCATTCCCTTTCAGTTCGCC
	R	CCTCATCTGGAGTGGTGTCATT
mN-Cadherin	F	AACTTGCCAGAAAACTCCAGA
	R	GCTGTATCTCAGGGAAAGGTT
mNOS3	F	AGCATCACCTACGACACCCT
	R	AGCGTCTTGAGGTACAGGGC
mSCF	F	GGCAAATCTTCCAAATGACTATATGA
	R	GCCAACAATGACTAGGCAAAACA
mSDF-1	F	GCCAACGTCAAGCATCTGAAAA
	R	TCTTCAGCCGTGCAACAATC
mTHPO	F	GGAACTCATGGGCTCTTTGC
	R	AAGCTCCGGGCGAGATGT
mVCAM1	F	GCCTCAACGGTACTTTGGATA
	R	TGGAGTCACCGATTTGAGCAAT
mVE-Cadherin	F	TCCTCTGCATCCTCACTATCACA
	R	GTAAGTGACCAACTGCTCGTGA
mVEGF-A	F	GTACCTCCACCATGCCAAGT
	R	TCTGCTCTCCTTCTGTCGTG

Human primers		
h18S	F	GCCCTATCAACTTTCGATGGT
	R	CCGGAATCGAACCCTGATT
h 2MG	F	TCGCTCCGTGGCCTTAGCTGT
	R	CTTTGGAGTACGCTGGATAGCCTCC
hHPRT	F	CTTTGCTTTCCTTGGTCAGGCAGT
	R	CGTGGGGTCCTTTTCACCAGCAA
hAngpt1	F	TGCCAGAACCCAAAAAGGTG
	R	TTACAGTCCAACCTCCCCCA
hAngpt2	F	GTGAGGATGGCAGCGTTGA
	R	AACAAACTCATTTCCCAGCCAA
hBMP4	F	AGGAGCTTCCACCACGAAGA
	R	GCAGAGTTTTCACTGGTCCCT
hCD31	F	GGAGTCCTGCTGACCCTTCTGCT
	R	CGTCCAGTCCGGCAGGCTCT
hCSF1	F	GATGGAGACCTCGTGCCAAA
	R	AGGTAGCACACTGGATCTTTCA
hCSF2	F	AGCCTCACCAAGCTCAAGGGC
	R	AGTTTCCGGGGTTGGAGGGCA
hCSF3	F	CCTTCGCCTCTGCTTTCCAG
	R	TAGAACGCGGTACGACACCT
hCXCR4	F	TGACTTGTGGGTGGTTGTGT
	R	CCAGGCAGGATAAGGCCAAC
hDHH	F	GATGAGGAGAACAGTGGAGCC
	R	GTTCACCCGCTCCTTACAAC
hDKK1	F	TCATAGCACCTTGGATGGGT
	R	GACCGGAGACAAACAGAACCT
hDll1	F	GGTGCTCTCGGCCTTGCTGTGT
	R	GTTGCGGTTCCCCAGCAGCC
hDll4	F	GTGGGTCAGAACTGGTTATTGGA
	R	TGCAGATGACCCGGTAAGAGT
hFGF1	F	GGGGAAATCACCACCTTCACA
	R	CTTGTAATTCCCTGGAGGCAGA
hFGF2	F	GCAAAAACGGGGGCTTCTT
	R	TGTAGCTTGATGTGAGGGTCG

hIGF1	F	GGTGGATGCTCTTCAGTTCGT
	R	TACCCTGTGGGCTTGTTGAAA
hIGFbp2	F	GCGAGGGCACTTGTGAGA
	R	TGAGTGGTCATCGCCATTGT
hIGFbp3	F	CAGTGTGGAGAGCCCGTCCG
	R	GGAGGGGGTGGAACTTGGGATCA
hIGFbp5	F	CTACCGCGAGCAAGTCAAGA
	R	GTAGGTCTCCTCGGCCATCT
hIL-3	F	GCTGGACTTCAACAACCTCA
	R	CTGTTGAATGCCTCCAGGTTTG
hIL-6	F	CTGAACCTTCCAAAGATGGCTG
	R	ACCAGGCAAGTCTCCTCATT
hJagged1	F	ACCGCAACCGCATCGT
	R	GCCTCCACAAGCAACGTATAGG
hJagged2	F	GTCGTCATCCCCTTCCAGTTC
	R	CTCATTCGGGGTGGTATCGTT
hN-Cadherin	F	TATCGAAGGATGTGCATGAAGG
	R	CAGGCTCACTGCTCTCATATTG
hNOS3	F	CATCCCTACTCCCACCAGCG
	R	ACCTCCCAGTTCTTCACACGA
hSCF	F	GTGGATGACCTTGTGGAGTGCGT
	R	GCCTGGGTTCTGGGCTCTTGA
hSDF-1	F	GCCAACGTCAAGCATCTCAAA
	R	TCTGTTGTTGTTCTTCAGCCG
hTHPO	F	CTTCGTGACTCCCATGTCCT
	R	CAGGTGTAGGCAAAGGGTGAA
hVCAM1	F	CCTGGGAAGATGGTCGTGAT
	R	TCTGGGGTGGTCTCGATTTT
hVE-Cadherin	F	TGAGTCGCAAGAATGCCAAGT
	R	CGGAAGACCTTGCCCACATA
hVEGF-A	F	GGAGGAGGGCAGAATCATCAC
	R	GGTCTCGATTGGATGGCAGT

RESULTS

Systemic anti-Dll4 treatment interferes with the BM vascular niche

We asked whether a therapeutic (systemic) approach of Dll4 blockade would affect the BM vascular niche. For that, we sub-lethally irradiated mice (300rad), therefore inducing myeloablation and BM turnover, and systemically treated them with a neutralizing anti-Dll4 antibody, HMD4-2 (Figure 2.1A).

We used six different vascular markers to characterize the effects of anti-Dll4 in the BM vascular niche: CD31, CD105 and VE-Cadherin antibodies, widely used to identify BM ECs [9,13]; VEGFR3 antibody, described as a specific marker of BM sinusoids [13]; SMA antibody, which labels pericytes in arteries and capillaries [23-25]; and Lycopersicon esculentum lectin, used as a pan-endothelial marker that stains perfused vessels [19,54].

By day 15 post-irradiation, increased number of CD31$^+$ and VE-Cadherin$^+$ vessels were scored in the BM of anti-Dll4 treated mice, with no significant changes in CD105$^+$, VEGFR3$^+$, SMA$^+$, and lectin$^+$ vessels (Figure 2.1B, C, S1A). VE-Cadherin mRNA expression was also increased in vivo and in vitro following anti-Dll4 treatment (Figure 2.2B, C).

Furthermore, anti-Dll4 treatment following myeloablation also increased BM megakaryocyte content (Figure 2.1D). Bone marrow endothelial VE-Cadherin expression had been associated with an increase in megakaryocyte numbers [55]. Moreover, it has been reported that Dll4 impairs the final stages of megakaryocytic differentiation, without affecting its early stages, also concordant with our data [34]. Therefore, the increase in megakaryocyte numbers herein described might be due to the increase in VE-Cadherin, to a direct effect of anti-Dll4 treatment on megakaryocytes, or both.

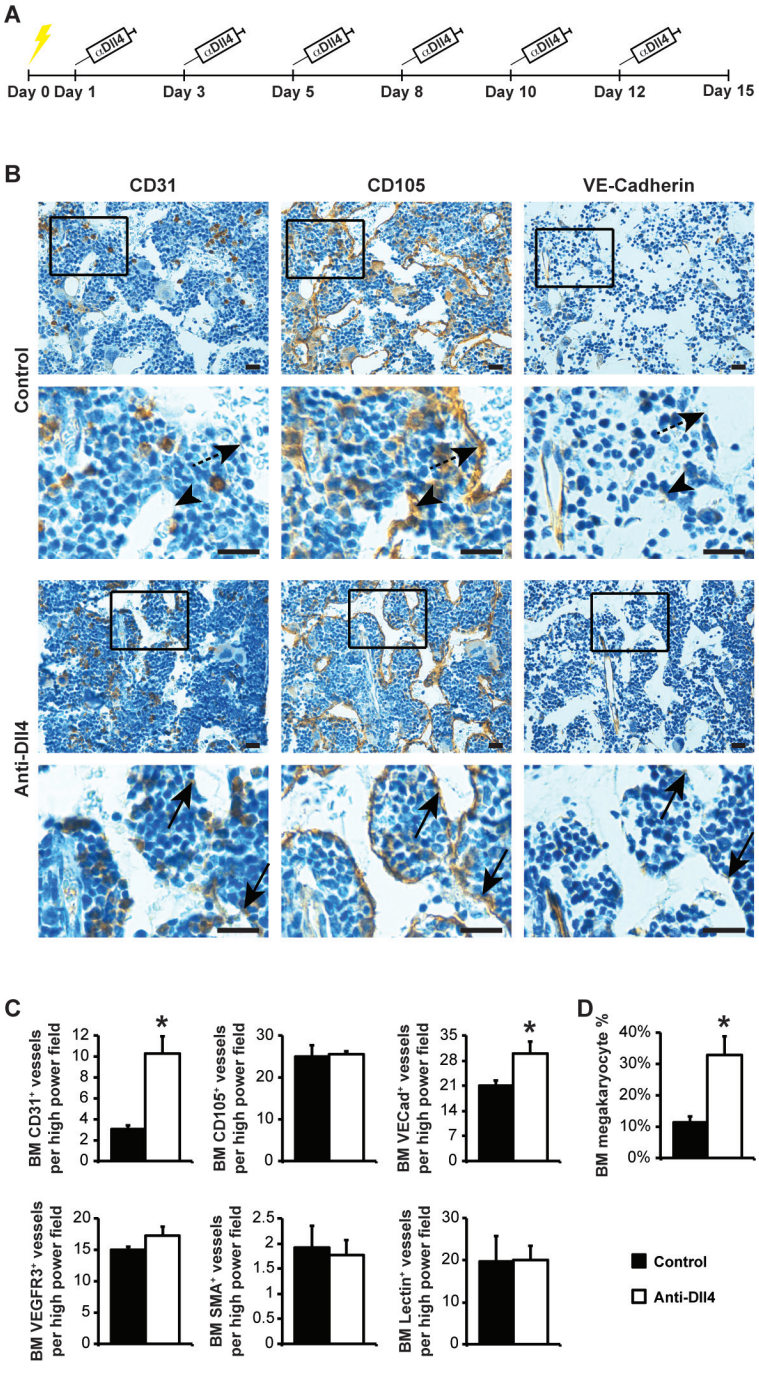


Figure 2.1. Therapeutic anti-Dll4 blockade interferes with the BM vascular niche.

A. Schematic representation of the clinical assessment of anti-Dll4 treatment. Yellow lightening bolt, sub-lethal irradiation.

B. Immunohistochemistry for CD31, CD105 and VE-Cadherin counterstained with Mayer's haemalum (Leica DMD 108). Sequential sections represent the same blood vessels. Arrowhead, $CD31^{-}CD105^{+}Ve\text{-}Cadherin^{+}$ blood vessel; dashed arrow, $CD31^{-}CD105^{+}VE\text{-}Cadherin^{-}$ blood vessel; arrow, $CD31^{+}CD105^{+}VE\text{-}Cadherin^{+}$ blood vessel. Bar=20μm.

C. CD31, CD105, VE-Cadherin, VEGFR3, SMA and Lectin-positive vessel count, per high power field (400x, Leica DMD 108), reveal an increase of CD31 and VE-Cadherin-positive BM vessels in anti-Dll4 treated mice.

D. Flow cytometric analysis of the percentage of megakaryocytes ($CD41^{+}$ cells) in the BM shows an increase of BM megakaryocyte cell percentage in anti-Dll4 treated mice.

Data are means ± s.e.m. *, $p<0.05$; data represents one of three experiments in which n=3.

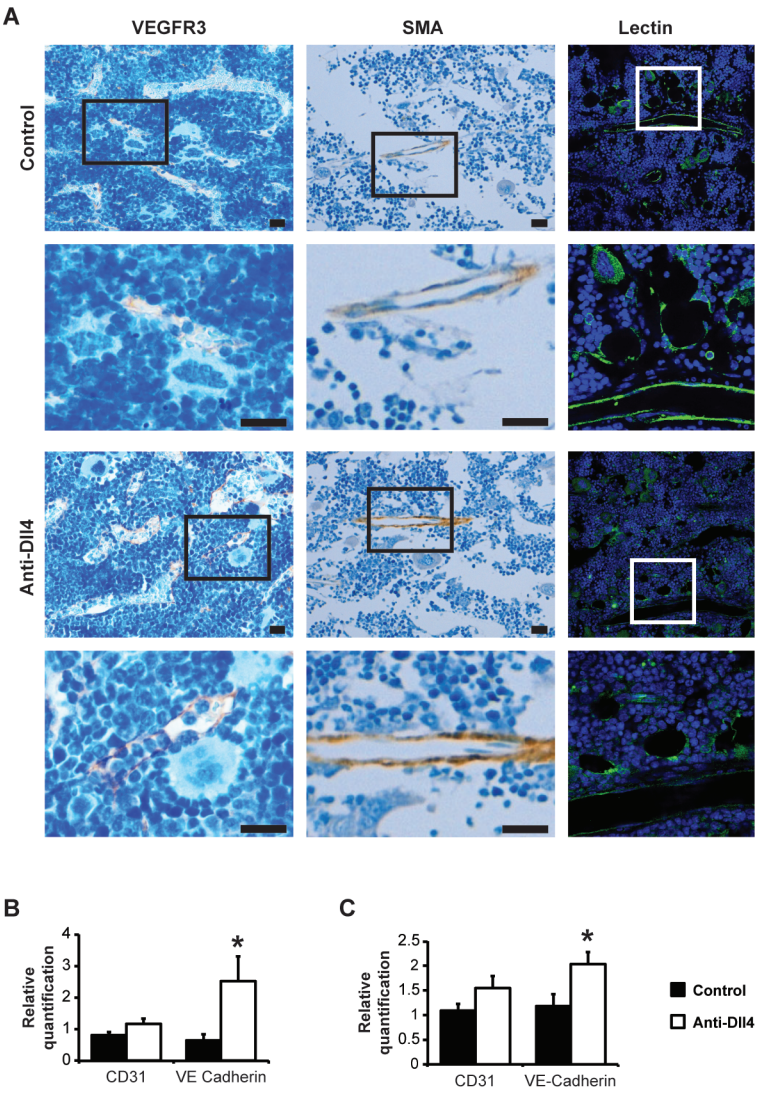


Figure 2.2. Anti-Dll4 blockade interferes with the BM vascular niche.
A. Immunohistochemistry for VEGFR3 and SMA counterstained with Mayer's haemalum (Leica DMD 108). Immunofluorescence for lectin (Leica LSM 510). Bar=20µm.
B. Relative quantification of mRNA from total BM reveals an increase in VE-Cadherin, but not CD31, expression in anti-Dll4 treated mice.
C. Relative quantification of mRNA from HUVEC reveals an increase in VE-Cadherin, but not CD31, expression in anti-Dll4 treated cells.
Data are means ± s.e.m. *, $p<0.05$; n=3.

These results were surprising, as previous work had shown quantitative vascular changes in tumors upon anti-Dll4 treatment [18,19]. However, in our study, we observed a qualitative modulation of the BM vascular niche (as suggested by the use of the different vascular markers). Therefore, we further characterized the type of blood vessels in the BM microenvironment. As previously described, we found VEGFR3 to be a specific sinusoidal marker [13], lectin to stain all types of blood vessels in the BM [54] (Figure 2.2A), and SMA to stain for pericyte-covered (stable) blood vessels, such as arteries and capillaries [24,25] (Figure 2.2A, 2.3a,c). CD31, CD105 and VE-Cadherin have been extensively used as BM EC markers [5,6,9,13], but the CD31 and VE-Cadherin specific modulation has led us to further

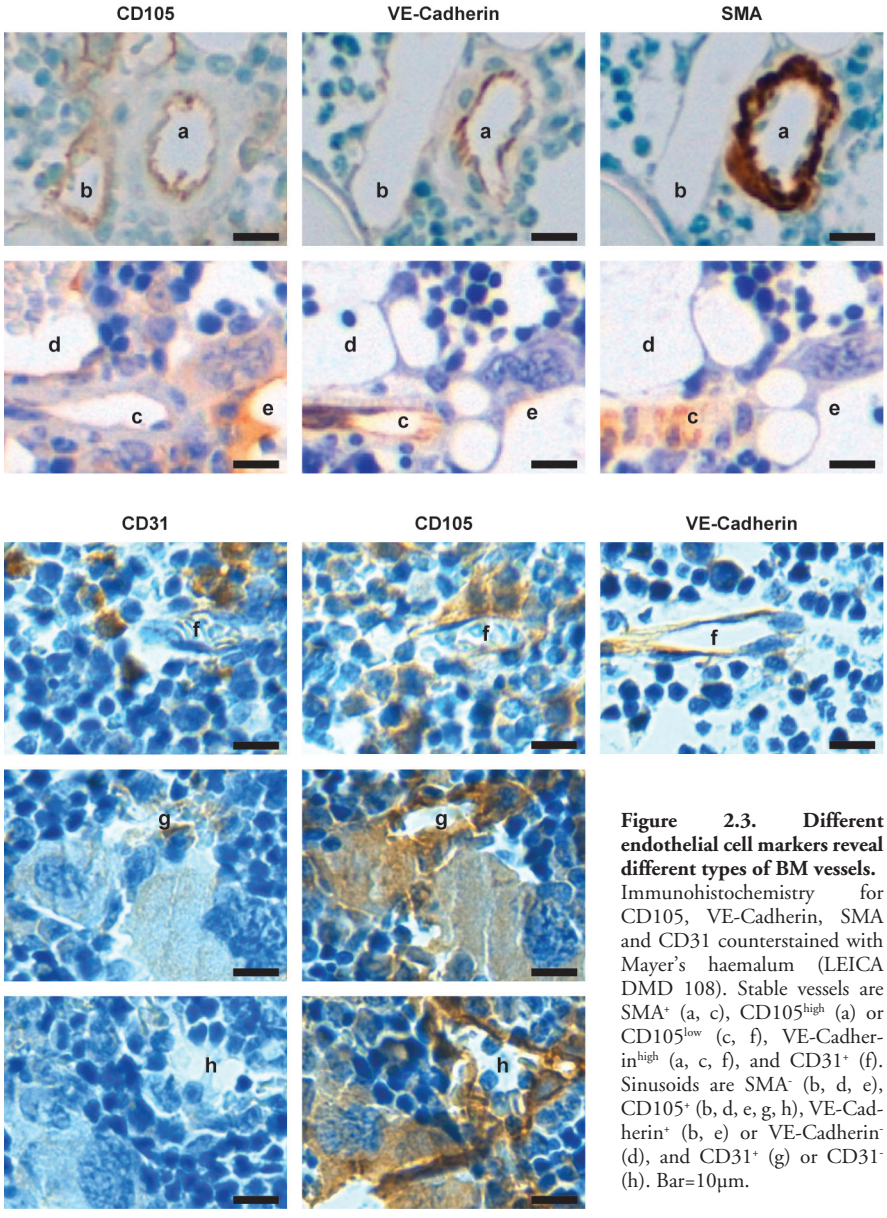


Figure 2.3. Different endothelial cell markers reveal different types of BM vessels. Immunohistochemistry for CD105, VE-Cadherin, SMA and CD31 counterstained with Mayer's haemalum (LEICA DMD 108). Stable vessels are SMA^{+} (a, c), $CD105^{high}$ (a) or $CD105^{low}$ (c, f), VE-$Cadherin^{high}$ (a, c, f), and $CD31^{+}$ (f). Sinusoids are SMA^{-} (b, d, e), $CD105^{+}$ (b, d, e, g, h), VE-$Cadherin^{+}$ (b, e) or VE-$Cadherin^{-}$ (d), and $CD31^{+}$ (g) or $CD31^{-}$ (h). Bar=10µm.

characterize these vessels. As shown in Figure 2.3, BM stable vessels are $CD105^{high/low}$, VE-$Cadherin^{high}$ and $CD31^{+}$, whereas BM sinusoids are $CD105^{+}$, VE-$Cadherin^{+/-}$, and $CD31^{+/-}$ in sub-lethally irradiated mice.

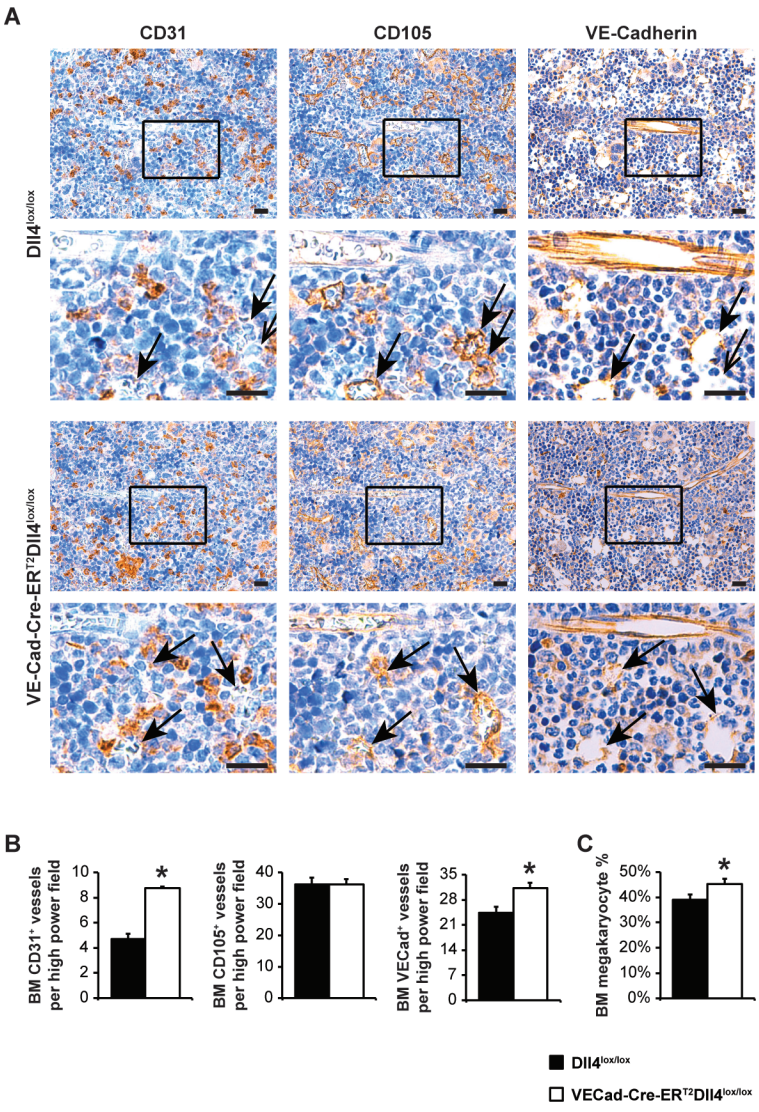


Figure 2.4. Endothelial cell-specific Dll4 blockade interferes with the BM vascular niche.

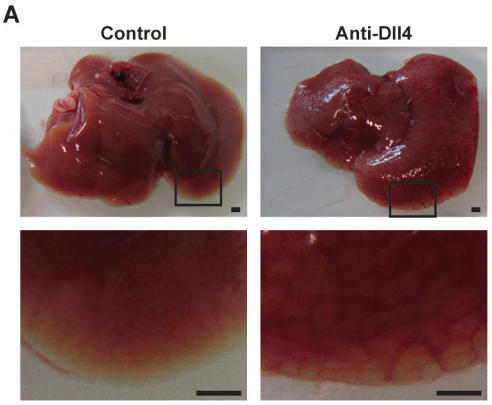
A. Immunohistochemistry for CD31, CD105 and VE-Cadherin counterstained with Mayer's haemalum (Leica DMD108). Bar=20µm.

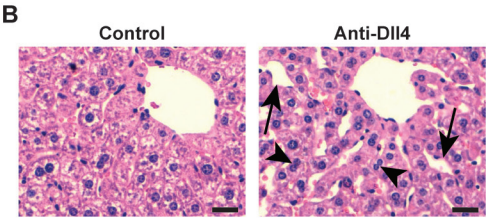
B. CD31, CD105 and VE-Cadherin-positive vessel count, per high power field (400x, Leica DMD108), reveal an increase of CD31 and VE-Cadherin-positive BM vessels in VECad-Cre-ER^{T2}Dll4$^{lox/lox}$ mice.

C. Flow cytometric analysis of the percentage of megakaryocytes (CD41^{+} cells) in the BM shows an increase of BM megakaryocyte cell percentage in mice.

Data are means ± s.e.m. *, p<0.05 ; n=11.

Next, we asked whether these BM vascular niche-specific changes were a direct effect of Dll4 blockade on the ECs. For that, we used inducible, conditional knockout (VECad-Cre-ER^{T2}Dll4$^{lox/lox}$) mice and assessed the number of CD31, CD105 and VE-Cadherin vessels, as well as the percentage of megakaryocytes in the BM. Consistent with the effects reported earlier (after systemic anti-Dll4 treatment), we observed a similar phenotype in this genetic targeting of Dll4, with an increase in CD31^{+} and VE-Cadherin^{+} vessels without modulation of CD105^{+} vessels, and an increase in the percentage of CD41^{+} megakaryocytes (Figure 2.4). These data suggest the effects of anti-Dll4 blockade in the BM vascular niche are exerted predominantly on VE-Cadherin-expressing BM ECs.





:h the

ti-Dll4

re cen-
regen-
hology

observed in control mice; hematoxilin-eosin staining (Leica DMD 108). Bar=25µm.
Data are means ± s.e.m. *, p<0.05 ; data represents one of three experiments in which n=3.

The BM vascular modifications herein described were accompanied by systemic defects in the vascular compartment of the liver (Figure 2.5), as previously reported by others [56].

Together, these data suggest systemic Dll4 blockade perturbs the BM vascular niche, favoring CD31$^+$ and VE-Cadherin$^+$ ECs expansion and increasing BM megakaryocyte content.

Specific effects of anti-Dll4 treatment on endothelial cells

Next, we investigated the mechanisms by which anti-Dll4 could affect EC function.

First, we characterized the BM endothelial phenotype induced by systemic anti-Dll4 blockade in more detail. We used a stem cell marker, c-kit, and found some BM vessels to be c-kit$^+$ (Figure 2.6A). C-kit is unappreciated as a BM vessel marker, despite *in vitro* reports of c-kit expression in BM primary ECs [57]. Some BM vessels were previously shown to express another stem cell marker, stem cell antigen-1 (Sca-1) [13], but its endothelial functions are still unknown. The overall percentage of c-kit$^+$ vessels (assessed from double labeling with CD105) also increased in anti-Dll4 treated animals (Figure 2.6A, B).

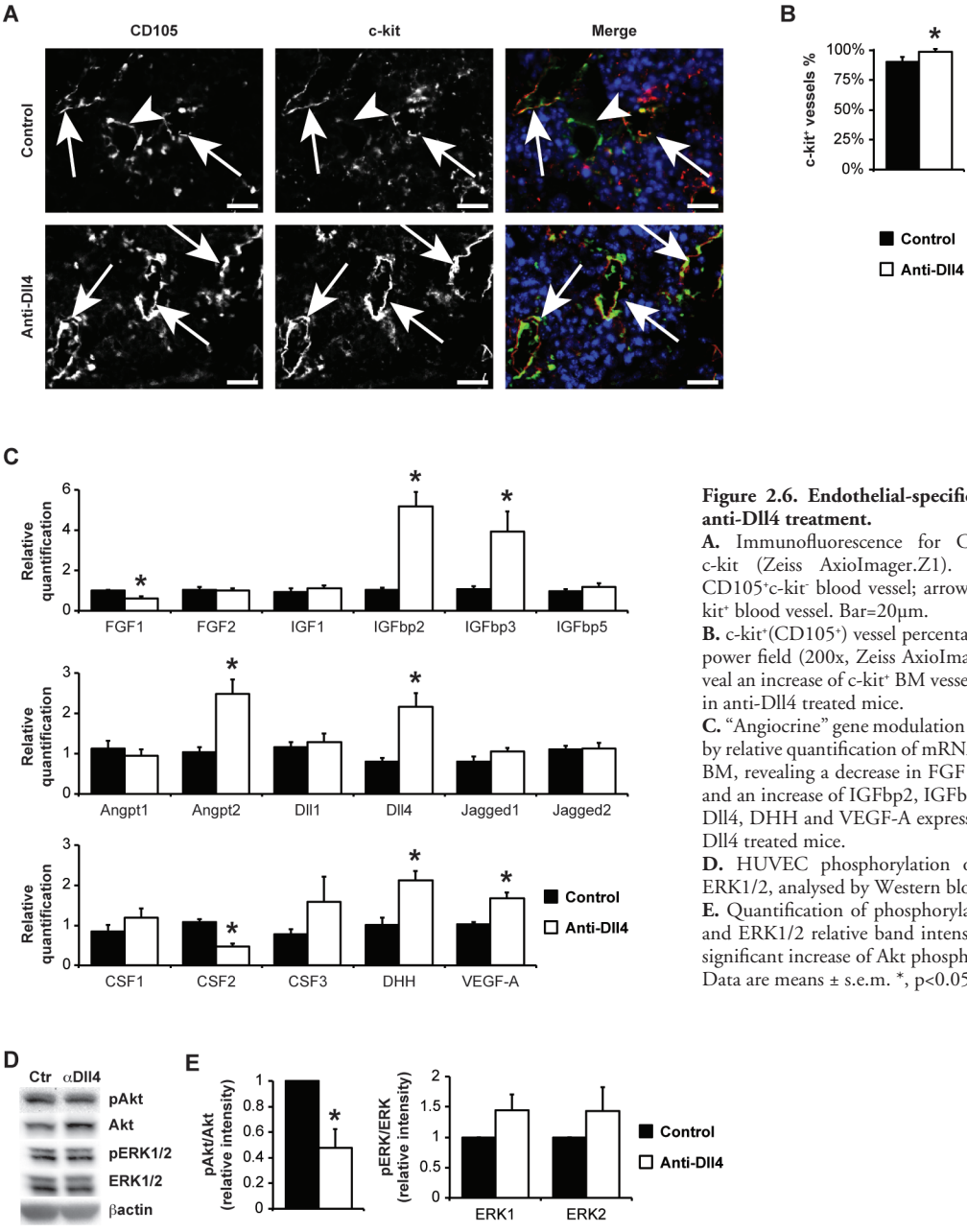


Figure 2.6. Endothelial-specific effects of anti-Dll4 treatment.

A. Immunofluorescence for CD105 and c-kit (Zeiss AxioImager.Z1). Arrowhead, $CD105^{+}$c-kit^{-} blood vessel; arrow, $CD105^{+}$c-kit^{+} blood vessel. Bar=20µm.

B. c-kit^{+}($CD105^{+}$) vessel percentage, per high power field (200x, Zeiss AxioImager.Z1), reveal an increase of c-kit^{+} BM vessel percentage in anti-Dll4 treated mice.

C. "Angiocrine" gene modulation was assessed by relative quantification of mRNA from total BM, revealing a decrease in FGF1 and CSF2 and an increase of IGFbp2, IGFbp3, Angpt2, Dll4, DHH and VEGF-A expression in anti-Dll4 treated mice.

D. HUVEC phosphorylation of Akt and ERK1/2, analysed by Western blotting.

E. Quantification of phosphorylation of Akt and ERK1/2 relative band intensity reveals a significant increase of Akt phosphorylation.

Data are means ± s.e.m. *, $p<0.05$; n=3.

Next, we searched for modulation of "angiocrine" genes and of MAPK and Akt signaling pathways in our system, since these were considered crucial for the instructive role exerted by the BM vascular niche in promoting hematopoietic recovery [16]. We performed qPCR analysis on a set of "angiocrine" genes, chosen because these are expressed depending on the activation state of BM ECs [16] and because of their involvement in hematopoietic recovery and vascular remodeling (Figure 2.6C, 2.7).

Anti-Dll4 treated animals showed a significant decrease in BM expression of fibroblast growth factor 1 (FGF1) and colony stimulating factor 2 (granulocyte-macrophage, CSF2) and an increase in insulin-like growth factor binding protein 2 (IGFbp2), IGFbp3, angiopoietin 2 (Angpt2), Dll4, desert hedgehog (DHH) and vascular endothelial growth factor A (VEGF-A) (Figure 2.6C, 2.7A).

This increase in VEGF-A (but not stromal-derived factor 1 alpha, SDF-1α, or stem cell factor, SCF) mRNA levels was accompanied by an increased VEGF-A protein levels in BM plasma, as assessed by ELISA (Figure 2.7B).

In order to identify endothelial-specific "angiocrine" gene modulation, we treated HUVEC *in vitro* with anti-Dll4 antibody. Anti-Dll4 treatment resulted in a significant decrease in FGF1 and CSF3, but not CSF2, and an increase in VEGF-A expression (Figure 2.7C). Genes whose expression was not changed *in vivo* were modulated *in vitro*, namely FGF2, CSF3, interleukin 6 (IL-6) and SCF (Figure 2.7C). Dll4 expression, however, was decreased *in vitro*, and increased *in vivo* (Figure 2.7C). The latter phenotypes can be interpreted as a non-EC-specific "angiocrine" gene modulation; another possibility is that the timing, activation state or EC identity of this *in vitro* assessment does not mimic BM ECs characteristics.

After characterizing "angiocrine" gene modulation, we searched for alterations of Akt and MAPK signaling pathways induced by anti-Dll4

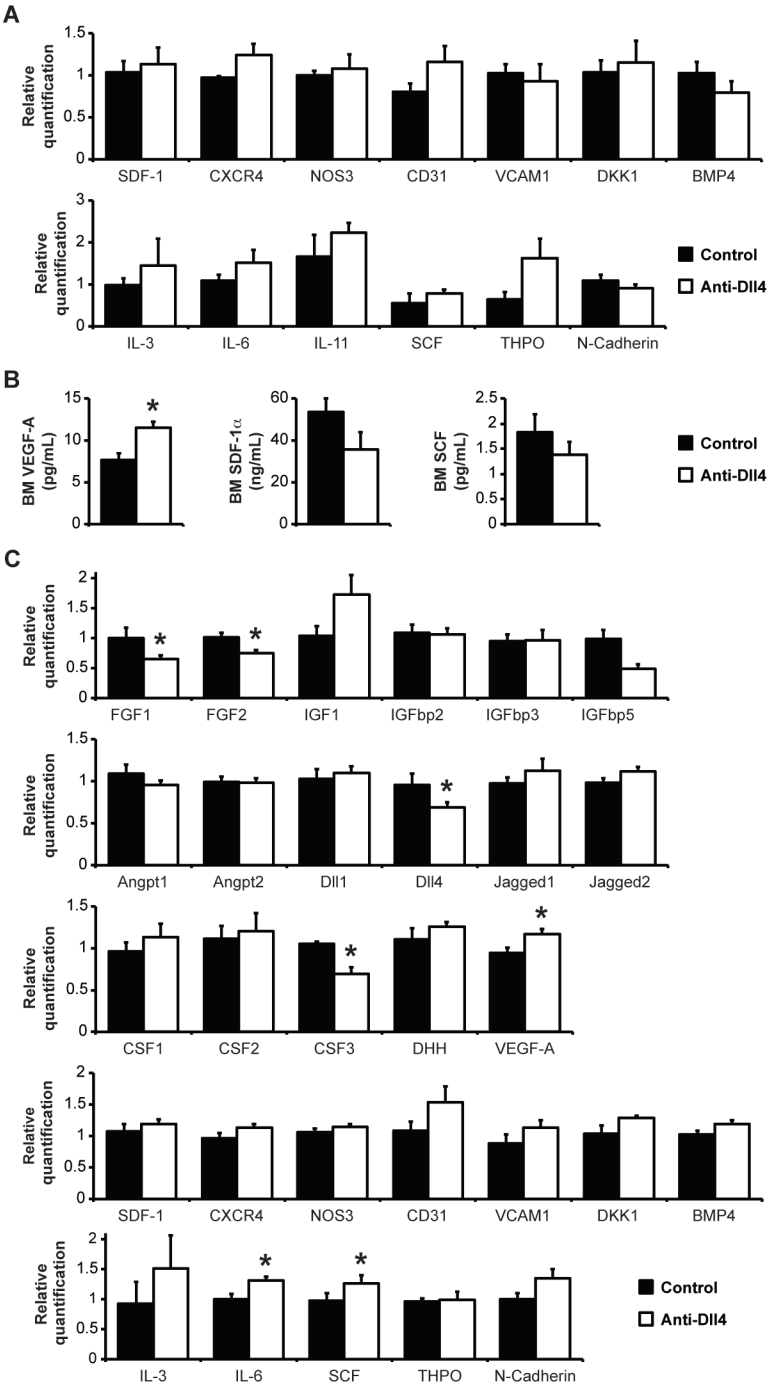


Figure 2.7. Endothelial-specific effects of anti-Dll4 treatment.
A. Angiocrine gene modulation was assessed by relative quantification of mRNA from total BM. None of the displayed genes is modulated by in vivo anti-Dll4 treatment.
B. Bone marrow VEGF-A, SDF-1α and SCF content, assessed by ELISA.
C. Angiocrine gene modulation was assessed in vitro by relative quantification of mRNA from HUVEC. HUVEC subjected to anti-Dll4 treatment decreases FGF1 and increases VEGF-A expression, similar to total BM from anti-Dll4 treated mice. CSF3, but not CSF2, expression is decreased upon in vitro anti-Dll4 treatment. FGF2 and Dll4 are significantly decreased, and IL-6 and SCF are significantly increased in anti-Dll4 treated cells.
Data are means ± s.e.m. *, $p<0.05$; n=3.

treatment. In light of the theory supported by Kobayashi *et al.*, the fine-tuning between Akt and MAPK activation in BM ECs balances self-renewal vs. differentiation of HSPCs. We found that treatment of HUVEC with anti-Dll4 decreased Akt phosphorylation, but did not induce significant changes in MAPK activation (Figure 2.6D, E), which supports the notion that reduced Akt and equal MAPK promotes the maintenance of the HSPCs pool [16].

These data suggest that modulating the BM vascular niche by anti-Dll4 treatment increases c-kit$^+$ vessels and affects BM ECs activation state and "angiocrine" factors production.

Anti-Dll4 treatment perturbs hematopoietic recovery following irradiation

Having shown systemic anti-Dll4 treatment affected BM ECs *in vivo* and *in vitro*, including "angiocrine" gene modulation, next we explored the hematopoietic effects of anti-Dll4 treatment in BM hematopoietic recovery following myeloablation.

Both BM and PB from anti-Dll4 treated mice showed increased myeloid cell content (CD11b$^+$) (Figure 2.8A). The BM lymphocytic compartment was also affected by the anti-Dll4 treatment; there was a significant decrease in both CD3$^+$ T and B220$^+$ B lymphocytes, with no significant changes in the PB (Figure 2.8A).

In contrast, anti-Dll4 treatment does not seem to affect BM progenitor cell populations. As shown in Figure 2.8B, there were no significant changes in the percentage of BM or PB EPCs (Sca1$^+$Flk1$^+$) or HSPCs (Sca1$^+$Flk1$^-$), with a trend for an increase of BM HSPCs ($p=0.07$) in anti-Dll4 treated mice.

After characterizing the global alterations in hematopoiesis upon anti-Dll4 treatment, we performed *in vitro* CFU assays, counting single-cell derived colonies, which represent either multipotent (CFU-granulo-

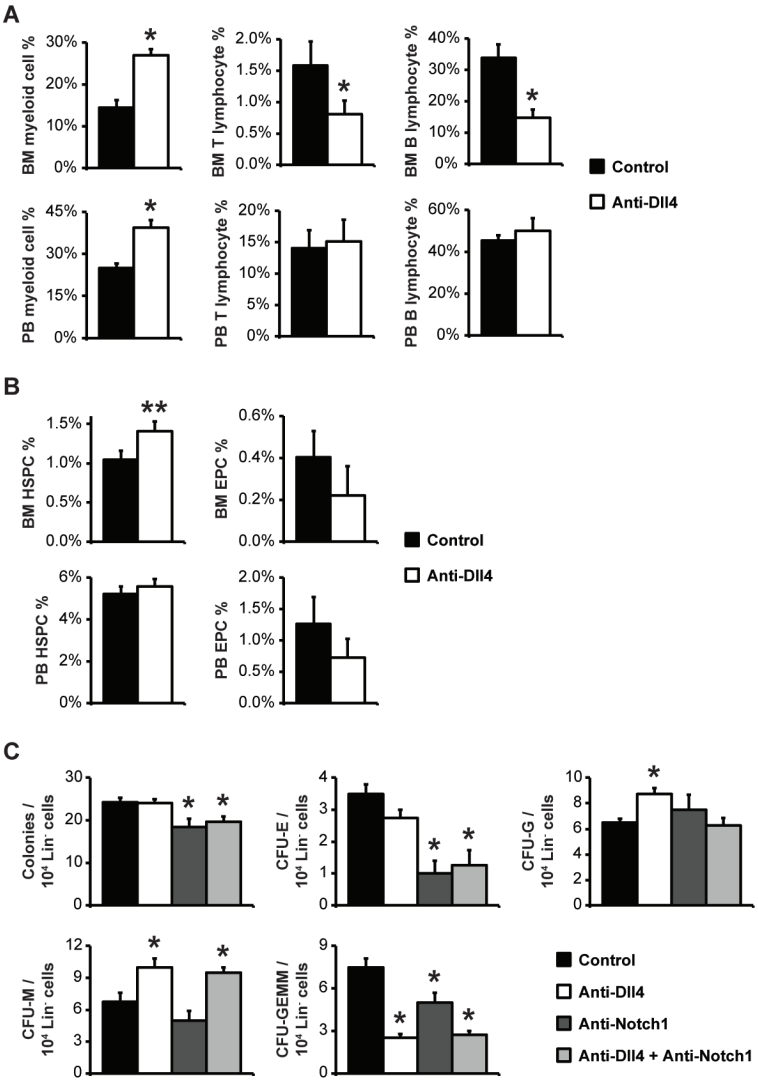


Figure 2.8. Anti-Dll4 treatment perturbs hematopoiesis following irradiation.

A. Flow cytometric analysis of the percentage of myeloid ($CD11b^+$) cells, T lymphocytes ($CD3^+$ cells), and B lymphocytes ($B220^+$) in the BM and PB, revealing an increase in both myeloid BM and PB content and a decrease in T and B lymphocyte BM content in anti-Dll4 treated mice.

B. Flow cytometric analysis of the percentage of stem/progenitor cells, namely HSPCs ($Sca1^+Flk1^-$) and EPCs ($Sca1^+Flk1^+$), revealing that anti-Dll4 treatment does not significantly affect these populations, notwithstanding the trend (p=0.07) towards and increase of BM HSPCs.

Data are means ± s.e.m. *, $p<0.05$, **, p=0.07 ; data represents one of three experiments in which n=3.

C. Colony counts from methylcellulose culture of Lin- cord blood-derived cells reveal anti-Dll4 treatment in vitro induces an increased HSPCs potential to differentiate to the myeloid lineage (CFU-G and CFU-M), an effect independent upon anti-Notch1 treatment. Anti-Notch1 treatment, independent of combined anti-Dll4 treatment, induces a decrease in HSPCs potential to differentiate to the erythrocytic lineage (CFU-E), and decreased HSPCs differentiation potential (total colony number). All treatments reduced multipotent HSPCs (CFU-GEMM).

Data are means ± s.e.m. *, $p<0.05$; n=4.

cyte-erythrocyte-macrophage-megakaryocyte, CFU-GEMM), bipotent (CFU-granulocyte-macrophage) or unipotent (CFU-monocyte, CGU-M, CFU-granulocyte, CFU-G, or CFU-erythrocyte, CFU-E) [47,48]. These assays allowed us to evaluate if the hematopoietic effects seen with anti-Dll4 treatment could also be due to direct effects on hematopoietic elements, namely in their differentiation capacity.

For that, we sorted BM HSPCs ($Lin^{-}Sca1^{+}$) from anti-Dll4 treated and control mice and cultured these in methylcellulose *in vitro* [47,48]. In accordance with the lack of change in HSPCs frequency seen after anti-Dll4 treatment (Figure 2.8B), this did not affect HSPCs CFU potential, or increased colony number (Figure 2.9).

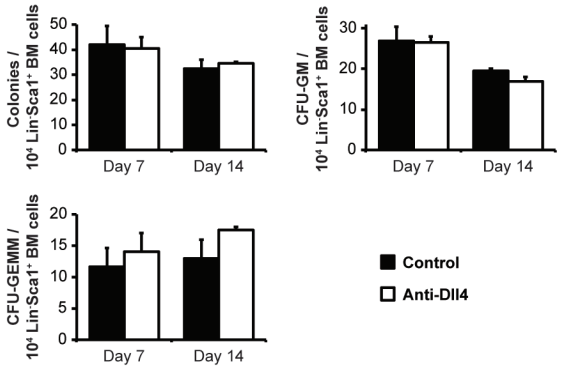


Figure 2.9. Anti-Dll4 treatment perturbs hematopoiesis following irradiation.
Colony counts from methylcellulose culture of $Lin^{-}Sca1^{+}$ sorted cells reveal anti-Dll4 treatment in vivo does not affect intrinsic stem cell's ability to differentiate into different hematopoietic lineages.
CFU-GM, Colony forming unit-granulocyte-macrophage.
CFU-GEMM, Colony forming unit-granulocyte-erythrocyte-macrophage-megakaryocyte.
Data are means ± s.e.m. *, $p<0.05$; n=3.

Next, we also assessed the direct effects of anti-Dll4 treatment on HSPCs, by treating naïve HSPCs with anti-Dll4 *in vitro*, in CFU assays. We further sought to determine whether Notch1 was the receptor involved in the possible hematopoietic changes, by blocking Notch1 using a monoclonal antibody either alone or in conjugation with anti-Dll4. We induced cord blood HSPCs' (Lin^{-}) differentiation in methylcellulose in the presence of either PBS, anti-Dll4, anti-Notch1, or the two neutralizing antibodies together. As shown in Figure 2.8C, anti-Dll4 treatment shifted differentiation towards the myeloid lineage (increased CFU-M and CFU-G colonies), an effect independent of anti-Notch1 treatment, as anti-Notch1 did not affect CFU-M or CFU-G colony number. Anti-Dll4 treatment reduced multipotent HSPCs (CFU-GEMM colonies), as did anti-Notch1 and the conjugation of both antibodies, indicating that anti-Dll4 treatment reduced multipotent HSPCs by reducing Notch1-mediated Notch signalling. Anti-Notch1, alone or combined with anti-Dll4, decreased HSPCs potential to differentiate to the erythroid lineage (CFU-E), and decreased HSPCs differentiation potential (total colony number). Both treatments reduced multipotent HSPCs (CFU-GEMM) (Figure 2.8C).

Taken together, these data suggest that besides affecting the BM vascular niche, anti-Dll4 treatment also perturbs hematopoietic cell differentiation and commitment.

Anti-Dll4 treatment of donor BM improves hematopoietic recovery following transplantation into lethally irradiated recipients

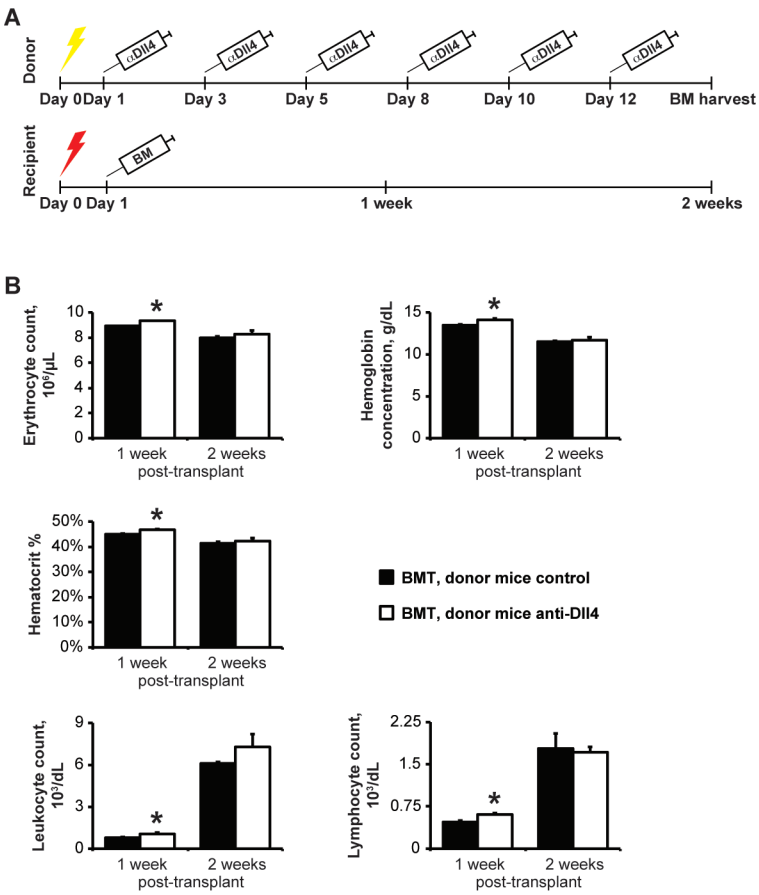


Figure 2.10. Anti-Dll4 treatment of donor BM improves hematopoietic recovery following transplantation into lethally irradiated recipients.
A. Schematic representation of the BMT. Yellow lightening bolt, sub-lethal irradiation; red lightening bolt, lethal irradiation.
B. Erythrocyte, hemoglobin, hematocrit, leukocyte and lymphocyte quantifications were assessed by PB cell blood counts. Data shows donor anti-Dll4 treated mice induces faster recovery of different hematological parameters day 1 week after transplantation.
Data are means ± s.e.m. *, $p<0.05$; n=3.

Next, we assessed whether the BM changes induced by anti-Dll4 treatment affected the efficiency of BM hematopoietic recovery in a transplant setting. For this purpose, we lethally irradiated recipient mice, which were subsequently transplanted with BM from untreated or anti-Dll4 treated mice (Figure 2.10A). Mice that received BM from anti-Dll4 treated mice showed evidence of improved hematopoietic recovery following lethal myeloablation (significantly faster recovery of leukocytes, hematocrit and lymphocytes), assessed by CBC (Figure 2.10B).

These data suggest that treatment of BM donor mice with anti-Dll4 improves hematopoietic recovery following lethal myeloablation.

DISCUSSION

The data presented in this paper show that systemic Dll4 blockade induces qualitative changes in the BM vasculature (which becomes more heterogeneous), which may be favorable in a BMT setting. A number of studies have proven the relevance of the BM vascular niche in hematopoiesis, but the heterogeneity of the BM vasculature has only been recently objectively assessed, clearly suggesting further detailed studies are needed to understand the importance of the different BM vessels for normal BM function [4-9,13,16,27,58-60].

We describe that systemic targeting Dll4, which has previously been shown to confine to particular vascular ECs (called "tip cells"), changes the vascular identity in the BM. Following myeloablation, we applied an anti-Dll4 treatment, similar to what is currently being performed in phase I clinical trials to treat patients with solid malignancies [21,22]. This treatment resulted in different vascular alterations in the BM, as shown by increased CD31, VE-Cadherin and c-kit$^+$ cells, without quantitative changes in CD105$^+$, VEGFR3$^+$, SMA$^+$ or lectin$^+$ vessels. The global BM vessel identity is therefore altered upon anti-Dll4 treatment.

Interestingly, CD31 is indispensable for several stages of hematopoiesis, EC survival and angiogenesis, which in turn are all crucial for hematopoietic recovery following myeloablation [13,27,61-67]. VE-Cadherin is also required for hematopoiesis and angiogenesis [55,68,69]. The role of c-kit in ECs, however, is unknown; studies assessing its role in angiogenesis and, more specifically, in the BM microenvironment, will be required for proper interpretation of the data presented in this paper.

Regarding the modulation of "angiocrine" genes, besides the increase in CD31$^+$ BM vessels previously described, we detected a significant increase in IGFbp2, IGFbp3, Angpt2, DHH and VEGF-A and a decrease in FGF1 and CSF2 expression in whole BM extracts from anti-Dll4 treated animals (Figure 2.6C). Even though FGF1, which is decreased upon anti-Dll4 treatment, prevents vessel regression, IGFbp3, Angpt2

and VEGF-A, which are increased, are modulators of vascular survival and re-growth, which, as previously mentioned, is crucially important for hematopoietic recovery following myeloablation [70-72].

Despite the decrease of CSF2, which is associated with a decrease in the myeloid lineage, IGF1 induces proliferation and differentiation of myeloid lineage cells [73], and DHH is important for granulocyte differentiation/proliferation in the BM [74]; moreover, the myeloid modulation we describe may be due to a direct effect of anti-Dll4 treatment on hematopoietic cells (Figure 2.8C). Both HSPCs and myeloid cells are reported to express Dll4 [32]. We show that anti-Dll4 treatment of HSPCs *in vitro* increases CFU-M and CFU-G number, independently of Notch1 modulation, but decreases multipotential progenitor cell-derived CFUs, similar to anti-Notch1 treatment (Figure 2.8C).

VEGF-A blocks both B and T lymphopoiesis [75-77]. The altered BM lymphocyte content observed might either be simply due to the increased myeloid content, to the VEGF-A increase in the BM, to the direct effect of anti-Dll4 in lymphoid cells, and/or to the effect of Dll4 inhibition in secondary hematopoietic organs, such as the thymus and spleen, which were previously shown to express Dll4 [35,49,56,78-80].

Regarding the signaling pathways that are proposed to trigger the EC role in hematopoiesis, we observed a decrease of Akt activation, without significant changes in MAPK (Erk1/2) after exposing ECs to anti-Dll4 treatment (Figure 2.6D, E). It should be noted that Dll4 has been shown to modulate the MAPK activation on a stimulus-depending manner [81]; the technical constrains to study EC-specific signaling pathways activation *in vivo* led us to an *in vitro* study, which may not completely mirror the *in vivo* systemic effects of anti-Dll4, nor the proper stimulus acting in different BM microenvironments.

In vivo assessments show both HSPC phenotype and function (reconstitution potential) were not impaired by systemic anti-Dll4 treatment,

and *in vitro* differentiation of HSPCs collected from the BM of anti-Dll4 treated mice was also not impaired, meaning HSPCs are unaffected by *in vivo* anti-Dll4 treatment (Figure 2.8B, 2.9 and 2.10B).

Systemic anti-Dll4 treatment in donor mice in a setting of BMT resulted in a mild, but significant, accelerated hematopoietic recovery of recipient mice (Figure 2.10) [7,13]. For a successful BMT, HSPCs must home and engraft in the BM, a process for which BM ECs are essential [11-13]. In this study, we transplanted whole BM mononuclear cells; this fraction includes BM ECs, which were previously shown to incorporate in the BM vasculature [12]. Interestingly, vascular CD31 and VE-Cadherin regulate the transition of HSPCs between blood and BM [65,68]. The increased VE-Cadherin and CD31-positive BM vessels from anti-Dll4 treated donor mice may have enhanced the homing of HSPCs in recipient mice, thereby leading to an overall faster hematopoietic recovery. Interestingly, we have also observed an increase in Dll4 expression in the BM of anti-Dll4 treated donor mice (Figure 2.6C). Given that anti-Dll4 treatment was performed only in donor mice, and not in recipients, the transplanted cells may have increased Dll4 protein levels. Remarkably, *in vitro* data have shown that increasing Dll4 signaling in HSPCs increases erythroid commitment and HSPCs proliferation, induces commitment and complete maturation to the T cell lineage, and maintains HSPCs stemness [32,34,82,83]. In our BMT model, these effects were transient, because the treatment was not maintained thoughout the process of hematopoietic recovery; therefore, the lymphoproliferative disease that mice overexpressing Dll4 in the hematopoietic lineage are expected to develop was not observed (evidenced by the long term survival of recipient mice) [33,35]. Alternatively, or in addition, IGFbp2 and IGFbp3 showed increased expression following anti-Dll4 treatment; these factors, by stabilizing IGF1, contribute towards the effects of anti-Dll4 treatment in promoting hematopoietic recovery following BMT [84].

Together, our data show that targeting Dll4 alters the vascular identity in the BM, mildly affects hematopoiesis, and promotes a faster hematopoietic recovery after BMT. We have characterized the BM vascular niche and provide evidence of its heterogeneity, which may create different microenvironments within the BM. This assessment may be particularly interesting to explore, as relevant information regarding the functional characterization of hematopoietic stem niches can be obtained. We further suggest anti-Dll4 blockade may be an interesting therapeutic approach in a BMT setting.

REFERENCES

1. Schofield, R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* 4, 7–25 (1978).

2. Zhang, J. *et al.* Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 425, 836–841 (2003).

3. Arai, F. *et al.* Tie2/Angiopoietin-1 Signaling Regulates Hematopoietic Stem Cell Quiescence in the Bone Marrow Niche. *Cell* 118, 149–161 (2004).

4. Kiel, M. J. *et al.* SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 121, 1109–1121 (2005).

5. Xie, Y. *et al.* Detection of functional haematopoietic stem cell niche using real-time imaging. *Nature* 457, 97–102 (2008).

6. Celso, Lo, C. *et al.* Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature* 457, 92–96 (2009).

7. Butler, J. M. *et al.* Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell* 6, 251–264 (2010).

8. Kimura, Y. *et al.* c-Kit-mediated functional positioning of stem cells to their niches is essential for maintenance and regeneration of adult hematopoiesis. *PLoS ONE* 6, e26918 (2011).

9. Ding, L., Saunders, T. L., Enikolopov, G. & Morrison, S. J. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 481, 457–462 (2012).

10. Rafii, S. *et al.* Human bone marrow microvascular endothelial cells support long-term proliferation and differentiation of myeloid and megakaryocytic progenitors. *Blood* 86, 3353–3363 (1995).

11. Kopp, H.-G. *et al.* Tie2 activation contributes to hemangiogenic regeneration after myelosuppression. *Blood* 106, 505–513 (2005).

12. Chute, J. P. *et al.* Transplantation of vascular endothelial cells mediates the hematopoietic recovery and survival of lethally irradiated mice. *Blood* 109, 2365–2372 (2007).

13. Hooper, A. T. *et al.* Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. *Cell Stem Cell* 4, 263–274 (2009).

14. Salter, A. B. *et al.* Endothelial progenitor cell infusion induces hematopoietic stem cell reconstitution in vivo. *Blood* 113, 2104–2107 (2009).

15. Lamorte, S., Remédio, L. & Dias, S. Communication between bone marrow niches in normal bone marrow function and during hemopathies progression. *Hematol Rep* 1, (2009).

16. Kobayashi, H. *et al.* Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nature Cell Biology* 12, 1046–1056 (2010).

17. Patel, N. S. *et al.* Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. *Cancer Research* 65, 8690–8697 (2005).

18. Noguera-Troise, I. *et al.* Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* 444, 1032–1037 (2006).

19. Ridgway, J. *et al.* Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature* 444, 1083–1087 (2006).

20. Real, C. *et al.* Bone marrow-derived endothelial progenitors expressing Delta-like 4 (Dll4) regulate tumor angiogenesis. *PLoS ONE* 6, e18323 (2011).

21. OncoMed Pharmaceuticals, Inc. A Phase 1 Dose Escalation Study of OMP-21M18 in Subjects With Solid Tumors. In: ClinicalTrials.gov. *Bethesda (MD): National Library of Medicine (US)* 1–3 (2013). at <http://clinicaltrials.gov/ct2/show/study/NCT00744562>

22. Regeneron PhamaceuticalsSanofi. A Multiple-Ascending-Dose Study of the Safety and Tolerability of REGN421(SAR153192) in Patients With Advanced Solid Malignancies. In: ClinicalTrials.gov. *Bethesda (MD): National Library of Medicine (US)* 1–3 (2013). at <http://clinicaltrials.gov/ct2/show/study/NCT00871559>

23. Skalli, O. *et al.* Alpha-smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericytes. *J. Histochem. Cytochem.* 37, 315–321 (1989).

24. Galmiche, M. C., Koteliansky, V. E., Brière, J., Hervé, P. & Charbord, P. Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway. *Blood* 82, 66–76 (1993).

25. Bianco, P., Riminucci, M., Gronthos, S. & Robey, P. G. Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells* 19, 180–192 (2001).

26. Bautch, V. L. Stem cells and the vasculature. *Nature Medicine* 17, 1437–1443 (2011).

27. Kopp, H.-G., Avecilla, S. T., Hooper, A. T. & Rafii, S. The bone marrow vascular niche: home of HSC differentiation and mobilization. *Physiology (Bethesda)* 20, 349–356 (2005).

28. Vainchenker, W. *et al.* Two monoclonal anti-platelet antibodies as markers of human megakaryocyte maturation: immunofluorescent staining and platelet peroxidase detection in megakaryocyte colonies and in in vivo cells from normal and leukemic patients. *Blood* 59, 514–521 (1982).

29. Kostyak, J. C., Naik, M. U. & Naik, U. P. Calcium- and integrin-binding protein 1 regulates megakaryocyte ploidy, adhesion, and migration. *Blood* 119, 838–846 (2012).

30. Lauret, E. *et al.* Membrane-bound delta-4 notch ligand reduces the proliferative activity of primitive human hematopoietic CD34+CD38low cells while maintaining their LTC-IC potential. *Leukemia* 18, 788–797 (2004).

31. Lahmar, M. *et al.* Distinct effects of the soluble versus membrane-bound forms of the notch ligand delta-4 on human CD34+CD38low cell expansion and differentiation. *Stem Cells* 26, 621–629 (2008).

32. Karanu, F. N. *et al.* Human homologues of Delta-1 and Delta-4 function as mitogenic regulators of primitive human hematopoietic cells. *Blood* 97, 1960–1967 (2001).

33. Dorsch, M. *et al.* Ectopic expression of Delta4 impairs hematopoietic development and leads to lymphoproliferative disease. *Blood* 100, 2046–2055 (2002).

34. Poirault-Chassac, S. *et al.* Notch/Delta4 signaling inhibits human megakaryocytic terminal differentiation. *Blood* 116, 5670–5678 (2010).

35. Yan, X. Q. *et al.* A novel Notch ligand, Dll4, induces T-cell leukemia/lymphoma when overexpressed in mice by retroviral-mediated gene transfer. *Blood* 98, 3793–

3799 (2001).

36. Hozumi, K. *et al.* Delta-like 4 is indispensable in thymic environment specific for T cell development. *Journal of Experimental Medicine* 205, 2507–2513 (2008).

37. Mohtashami, M. *et al.* Direct comparison of Dll1- and Dll4-mediated Notch activation levels shows differential lymphomyeloid lineage commitment outcomes. *The Journal of Immunology* 185, 867–876 (2010).

38. Todd, R. F., Nadler, L. M. & Schlossman, S. F. Antigens on human monocytes identified by monoclonal antibodies. *J. Immunol.* 126, 1435–1442 (1981).

39. Wang, J. *et al.* A Differentiation Checkpoint Limits Hematopoietic Stem Cell Self-Renewal in Response to DNA Damage. *Cell* 148, 1001–1014 (2012).

40. Coffman, R. L. & Weissman, I. L. B220: a B cell-specific member of th T200 glycoprotein family. *Nature* 289, 681–683 (1981).

41. Reinherz, E. L., Kung, P. C., Goldstein, G., Levey, R. H. & Schlossman, S. F. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. *Proc. Natl. Acad. Sci. U.S.A.* 77, 1588–1592 (1980).

42. Spangrude, G. J., Heimfeld, S. & Weissman, I. L. Purification and characterization of mouse hematopoietic stem cells. *Science* 241, 58–62 (1988).

43. Christensen, J. L. & Weissman, I. L. Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 98, 14541–14546 (2001).

44. Asahara, T. *et al.* Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275, 964–967 (1997).

45. Takahashi, T. *et al.* Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nature Medicine* 5, 434–438 (1999).

46. Wheat, L. A. *et al.* Acrolein inhalation prevents vascular endothelial growth factor-induced mobilization of Flk-1+/Sca-1+ cells in mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* 31, 1598–1606 (2011).

47. Nakahata, T. & Ogawa, M. Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential hemopoietic colonies. *Proc. Natl. Acad. Sci. U.S.A.* 79, 3843–3847 (1982).

48. Broxmeyer, H. E. *et al.* Hematopoietic stem/progenitor cells, generation of induced pluripotent stem cells, and isolation of endothelial progenitors from 21- to 23.5-year cryopreserved cord blood. *Blood* 117, 4773–4777 (2011).

49. Ishida, W. *et al.* Regulation of experimental autoimmune uveoretinitis by anti-delta-like ligand 4 monoclonal antibody. *Invest. Ophthalmol. Vis. Sci.* 52, 8224–8230 (2011).

50. Bassil, R. *et al.* Notch Ligand Delta-Like 4 Blockade Alleviates Experimental Autoimmune Encephalomyelitis by Promoting Regulatory T Cell Development. *The Journal of Immunology* 187, 2322–2328 (2011).

51. Sunamura, M. & Yagita, H. Neutralizing monoclonal antibody against human Dll4. (2008).

52. Sunamura, M. & Yagita, H. Anti-Dll4 binding protein. *UK Patent Application* 1–85 (2008).

53. Sekine, C. *et al.* Differential regulation of osteoclastogenesis by Notch2/Delta-like 1 and Notch1/Jagged1 axes. *Arthritis Research & Therapy* 14, R45 (2012).

54. Nakamura-Ishizu, A., Morikawa, S., Shimizu, K. & Ezaki, T. Characterization of sinusoidal endothelial cells of the liver and bone marrow using an intravital lectin injection method. *J. Mol. Histol.* 39, 471–479 (2008).

55. Avecilla, S. T. *et al.* Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nature Medicine* 10, 64–71 (2004).

56. Yan, M. *et al.* Chronic DLL4 blockade induces vascular neoplasms. *Nature* 463, E1–E1 (2010).

57. Candal, F. J. *et al.* BMEC-1: a human bone marrow microvascular endothelial cell line with primary cell characteristics. *Microvasc. Res.* 52, 221–234 (1996).

58. Takagi, S. *et al.* Membrane-bound human SCF/KL promotes in vivo human hematopoietic engraftment and myeloid differentiation. *Blood* (2012). doi:10.1182/

blood-2011-05-353201

59. Pitchford, S. C., Lodie, T. & Rankin, S. M. VEGFR1 stimulates a CXCR4-dependent translocation of megakaryocytes to the vascular niche, enhancing platelet production in mice. *Blood* 120, 2787–2795 (2012).

60. Li, X. M., Hu, Z., Jorgenson, M. L. & Slayton, W. B. High Levels of Acetylated Low-Density Lipoprotein Uptake and Low Tyrosine Kinase With Immunoglobulin and Epidermal Growth Factor Homology Domains-2 (Tie2) Promoter Activity Distinguish Sinusoids From Other Vessel Types in Murine Bone Marrow. *Circulation* 120, 1910–1918 (2009).

61. Vaporciyan, A. A. *et al.* Involvement of platelet-endothelial cell adhesion molecule-1 in neutrophil recruitment in vivo. *Science* 262, 1580–1582 (1993).

62. Piali, L. *et al.* CD31/PECAM-1 is a ligand for alpha v beta 3 integrin involved in adhesion of leukocytes to endothelium. *The Journal of Cell Biology* 130, 451–460 (1995).

63. Wu, Y., Welte, T., Michaud, M. & Madri, J. A. PECAM-1: a multifaceted regulator of megakaryocytopoiesis. *Blood* 110, 851–859 (2007).

64. Dhanjal, T. S. *et al.* A novel role for PECAM-1 in megakaryocytokinesis and recovery of platelet counts in thrombocytopenic mice. *Blood* 109, 4237–4244 (2007).

65. Ross, E. A. *et al.* A Novel Role for PECAM-1 (CD31) in Regulating Haematopoietic Progenitor Cell Compartmentalization between the Peripheral Blood and Bone Marrow. *PLoS ONE* 3, e2338 (2008).

66. Bird, I. N. *et al.* Homophilic PECAM-1(CD31) interactions prevent endothelial cell apoptosis but do not support cell spreading or migration. *Journal of Cell Science* 112 (Pt 12), 1989–1997 (1999).

67. Cao, G. *et al.* Involvement of human PECAM-1 in angiogenesis and in vitro endothelial cell migration. *Am. J. Physiol., Cell Physiol.* 282, C1181–90 (2002).

68. van Buul, J. D. *et al.* Migration of human hematopoietic progenitor cells across bone marrow endothelium is regulated by vascular endothelial cadherin. *J. Immunol.* 168, 588–596 (2002).

69. Nelson, C. M. & Chen, C. S. VE-cadherin simultaneously stimulates and inhibits cell proliferation by altering cytoskeletal structure and tension. *Journal of Cell Science* 116, 3571–3581 (2003).

70. Chang, K.-H. *et al.* IGF binding protein-3 regulates hematopoietic stem cell and endothelial precursor cell function during vascular development. *Proc. Natl. Acad. Sci. U.S.A.* 104, 10595–10600 (2007).

71. Lofqvist, C. *et al.* IGFBP3 suppresses retinopathy through suppression of oxygen-induced vessel loss and promotion of vascular regrowth. *Proc. Natl. Acad. Sci. U.S.A.* 104, 10589–10594 (2007).

72. Maisonpierre, P. C. *et al.* Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277, 55–60 (1997).

73. Heemskerk, V. H., Daemen, M. A. & Buurman, W. A. Insulin-like growth factor-1 (IGF-1) and growth hormone (GH) in immunity and inflammation. *Cytokine Growth Factor Reviews* 10, 5–14 (1999).

74. Lau, C.-I. *et al.* Regulation of murine normal and stress-induced erythropoiesis by Desert Hedgehog. *Blood* 119, 4741–4751 (2012).

75. Ohm, J. E. *et al.* VEGF inhibits T-cell development and may contribute to tumor-induced immune suppression. *Blood* 101, 4878–4886 (2003).

76. Huang, Y. *et al.* Distinct roles of VEGFR-1 and VEGFR-2 in the aberrant hematopoiesis associated with elevated levels of VEGF. *Blood* 110, 624–631 (2007).

77. Fragoso, R. *et al.* VEGF signaling on hematopoietic precursors restricts B-lymphoid commitment in vitro and in vivo. *Exp. Hematol.* 36, 1329–1336 (2008).

78. Shutter, J. R. *et al.* Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes & Development* 14, 1313–1318 (2000).

79. Itoi, M., Tsukamoto, N. & Amagai, T. Expression of Dll4 and CCL25 in Foxn1-negative epithelial cells in the post-natal thymus. *International Immunology* 19, 127–132 (2006).

80. Koch, U. *et al.* Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *Journal of Experimental Medicine* 205, 2515–2523 (2008).

81. Harrington, L. S. *et al.* Regulation of multiple angiogenic pathways by Dll4 and Notch in human umbilical vein endothelial cells. *Microvasc. Res.* 75, 144–154 (2008).

82. Dando, J. S. *et al.* Notch/Delta4 Interaction in Human Embryonic Liver CD34 +CD38 –Cells: Positive Influence on BFU-E Production and LTC-IC Potential Maintenance. *Stem Cells* 23, 550–560 (2005).

83. La Coste, de, A. *et al.* In vivo and in absence of a thymus, the enforced expression of the Notch ligands delta-1 or delta-4 promotes T cell development with specific unique effects. *J. Immunol.* 174, 2730–2737 (2005).

84. Tian, Z. G. *et al.* Recombinant human growth hormone promotes hematopoietic reconstitution after syngeneic bone marrow transplantation in mice. *Stem Cells* 16, 193–199 (1998).

3

LEUKEMIA CELL CYCLE ARREST INDUCED BY DELTA-LIKE 4 (DLL4)-INDEPENDENT NOTCH SIGNALING INHIBITION

In preparation. Remédio L., Yagita H. and Dias S.

CONTENTS

ABSTRACT **84**

INTRODUCTION **85**

METHODS **87**

Cell culture 87

Western blotting 87

Apoptosis assay 88

Cell cycle analysis 88

Statistical analysis 89

RESULTS **90**

Several leukemia and lymphoma cell lines express Dll4 90

Notch signaling pathway does not affect spontaneous leukemia cell apoptosis 91

Notch signaling pathway promotes leukemia cell growth arrest in a Dll4 independent manner 94

DISCUSSION **97**

REFERENCES **98**

ABSTRACT

Delta-like 4 (Dll4) is a ligand of the Notch pathway family which has been widely studied in the context of tumor angiogenesis. Delta-like 4 blockade was shown to result in non-productive angiogenesis and halted tumor growth, both in solid and hematological tumors. As Dll4 inhibitors enter the clinic for the treatment of solid tumors, there is an emerging need to explore its role in leukemia/lymphoma progress. We show several leukemia/lymphoma cell lines express Dll4. The role of Dll4:Notch signaling in these cells had not previously been assessed. Focusing in three different leukemia cell lines, HL-60, 697 and MOLT-4 as models of acute myeloid leukemia, B- and T-acute lymphoblastic leukemia, respectively, we report the role of Dll4:Notch signaling pathway in leukemia cell survival and growth. We show that up to 48 hours of treatment with recombinant human Dll4 (rhDll4), an anti-Dll4 neutralizing antibody or γ-secretase inhibitor the leukemia cell survival remains unaltered. Notch signaling pathway blockade, through γ-secretase inhibition, results in leukemia cell growth arrest, an effect independent of Dll4. Together, our data show that Dll4 is expressed by leukemia/lymphoma cell lines which is not involved in leukemia cell survival or growth.

INTRODUCTION

The mammalian Notch signaling pathway is composed of four receptors, Notch 1-4, and five ligands, Delta-like (Dll) 1, 3 and 4, and Jagged-1 and -2 [1]. Notch activating mutations are prevalent in approximately 50% of acute T lymphoblastic leukemia/lymphoma (T-ALL) cases [2-4]. Aggressive T cell leukemia mouse models were established by Notch1 and Notch3 constitutive activation, and also Dll4 overexpression [5-8]. However, the Dll4 involvement in the progression of different types of leukemias has been poorly assessed.

Similar to solid tumor models, Dll4 blockade affects hematological tumor angiogenesis and growth [9-15].

Delta-like 4-mediated Notch signaling was primarily described as a key regulatory factor for endothelial cell sprouting and angiogenesis [16], and anti-Dll4 neutralizing antibodies are currently being tested in clinical trials to target blood vessels in solid tumors [17,18]. In fact, we have previously shown that transplantation of bone marrow-derived $Dll4^{+/-}$ vascular progenitor cells into solid leukemia-bearing mice, using an acute myeloid leukemia (AML) cell line, increases the tumor vascular density and reduces the tumor size [14]. An important relationship between endothelial-specific Dll4 levels and tumor dormancy was established in tumor xenografts composed by T-ALL cell lines [12,13,15]. Furthermore, Dll4 is overexpressed in human AML bone marrow and is associated with higher angiogenesis and poor prognosis [19,20]. However, Dll4 blockade does not only affect tumor angiogenesis, but also the tumor cells [21,22] It remains unclear whether tumor growth suppression upon Dll4 blockade was due mostly to its microenvironmental effects, or also to a direct influence upon hematological tumor cells. This led us to investigate whether leukemia/lymphoma cells express Dll4, and whether Dll4 targeting directly affects different leukemia cell lines' survival (apoptosis) or proliferation (dormancy), related to the disease progression.

Apoptotic cell death is accompanied by the loss of phospholipid asymmetry of the plasma membrane without affecting the membrane integrity, with surface exposure of phosphatidylserine [23-25]. Late stage apoptosis involves the loss of the cell membrane integrity, thus permeabilizing the cells [25]. These characteristics are used to identify early and late stage apoptosis. Flow cytometry of single cell suspensions, using a combination of the phosphatidylserine binding protein Annexin V [26-30] and the DNA dye 7-aminoactinomycin D (7-AAD) [31], allow the identification of three cell populations: living cells (Annexin $V^-7\text{-}AAD^-$), early apoptotic cells (Annexin $V^+7\text{-}AAD^-$), and late apoptotic cells (Annexin$V^+7\text{-}AAD^+$) [32].

Cell proliferation can be measured by analyzing the cell cycle phase the cells are at, proportional to its DNA content. Highly proliferative cell populations typically exhibit higher percentage of cells in S and G_2/M phases, whereas more quiescent cell populations are longer in the G_0/G_1 phase, thus corresponding to a higher cell percentage in that phase of the cell cycle. This can be measured in single cell suspensions by flow cytometry, using fixed cells stained with the DNA dye propidium iodide (PI) and measuring the relative amount of DNA in each cell [33,34].

We have identified Dll4 expression in several leukemia/lymphoma cell lines. Our studies show that Dll4-mediated Notch signaling increase, through recombinant human Dll4 (rhDll4), or inhibition, through an anti-Dll4 neutralizing antibody, do not affect the proliferation or survival of myeloid, B- and T- acute leukemia cell lines. Moreover, we show that Notch signaling inhibition, through the γ-secretase inhibitor DAPT, reduces leukemia cell proliferation. Together, we show that several leukemia/lymphoma cell lines express Dll4, but modulating Dll4 levels *in vitro* does not affect leukemia cell survival or proliferation.

METHODS

Cell culture

Acute promyelocytic leukemia (AML), HL-60 [35], pre-B acute lymphoblastic leukemia (B-ALL), 697 [36], T acute lymphoblastic leukemia (T-ALL), MOLT-4 [37], erythroleukemia, HEL [38], B-prolymphocytic leukemia, JVM-13 [39], B cell non-Hodgkin's lymphoma, DoHH2 [40], Burkitt's lymphoma, Raji [41], and B-ALL, RCH-ACV [42]. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, 2mM L-Glutamine, antibiotic-antimycotic plus 10% heat-inactivated fetal bovine serum (FBS) (all from Gibco Invitrogen, Carlsbad, CA).

Western blotting

Cells were lysed with RIPA buffer (20mM Tris pH 7.5, 150mM NaCl, 5mM KCl, 5mM MgCl, 1% Triton X-100, protease inhibitor cocktail and 1mM sodium orthovanadate), and equal amounts of proteins were subjected to SDS–polyacrylamide gel electrophoresis with 12% Mini-Protean TGX precast gel (BioRad, USA). Proteins were transferred onto nitrocellulose membrane (Hybond-C Extra, GE Healthcare Life Sciences, Roosendaal, Netherlands) and subjected to standard immunoblotting. Briefly, membranes were blocked in tris-buffered saline (TBS) 0.1% Tween (TBST) (Sigma-Aldrich, Germany) containing 5% milk at room temperature for 1 hour and then incubated with the primary antibody – polyclonal rabbit anti-Dll4 antibody (Abcam, Cambridge, UK), 1:1000, or monoclonal mouse anti-β-actin (Sigma-Aldrich, Germany), 1:5000 – overnight at 4°C. The primary antibody was detected by incubation with horse radish peroxidase (HRP)-coupled secondary antibodies (ThermoScientific, Pittsburgh, USA) for 2 hours at room temperature. The chemiluminiscence detection was performed using SuperSignal West Pico Chemiluminiscent Substrate (ThermoScientific, Pittsburgh, USA). The membranes were visualized using ChemiDoc XRS+ Imaging System (BioRad, USA). The protein bands were quantified using ImageJ software (NIH, USA).

Apoptosis assay

Cells were cultured in 24-well plates (Corning, NY, USA) at a density of 5.10^5 cells/mL in serum-free RPMI, and starved overnight to induce cell cycle syncronization. To stimulate Dll4-mediated Notch signaling, cells were treated with soluble recombinant human Dll4 (rhDll4) (R&D Systems, Abingdon, UK) [43-45], 500ng/mL; equal quantity of phosphate-buffered saline (PBS) (Lonza, Walkersville, MD) 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, Germany) was added to the controls. To inhibit Dll4-mediated Notch signaling, cells were treated with neutralizing anti-human Dll4 antibody (MHD4-46) [46,47], 50 µg/mL; equal quantity of PBS was added to the controls. To inhibit Notch signaling, cells were treated with N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (Sigma-Aldrich, Germany) [44,48,49], 10µM; equal quantity of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) was added to the controls. After 24 or 48 hours of culture, the cells were harvested, washed twice in PBS, then ressuspended in annexin V staining buffer (0.01M Hepes buffer pH 7.4, 0.14M NaCl and 2.5mM $CaCl_2$) and incubated with Annexin-V-specific antibody-FITC and 7-AAD (both from Becton Dickinson, Franklin Lakes, NJ) for 15min. Flow cytometry was performed on FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Cell cycle analysis

Cells were cultured in 24-well plates (Corning, NY, USA) at a density of 5.10^5 cells/mL in serum-free RPMI, and starved overnight to induce cell cycle syncronization. To stimulate Dll4-mediated Notch signaling, cells were treated with rhDll4 (R&D Systems, Abingdon, UK), 500ng/mL; equal quantity of PBS 0.1% BSA was added to the controls. To inhibit Dll4-mediated Notch signaling, cells were treated with MHD4-46, 50 µg/mL; equal quantity of PBS was added to the controls. To inhibit Notch signaling, cells were treated with DAPT, 10µM; equal quantity of DMSO was added to the controls. After 24 or 48 hours of culture, the cells were harvested, washed in PBS, and fixed in ice-cold 70% ethanol at -20°C for at least 48h. Cells were then centrifuged at 2000rpm, washed twice in PBS, and ressuspended in a solution of 50µg/mL propidium iodide (Sigma-Aldrich, Germany) plus 100µg/mL RNAseA (Citogene). Cells were incubated 3h at 4°C. Flow cytometry was performed on FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA) using the Dean-Jett-Fox model.

Statistical analysis

Results are expressed as mean ± standard error. The data analysis was performed with Prism 4 (GraphPad software, LaJolla, CA, USA) using Mann Whitney U test. P values of <0.05 were considered statistically significant.

RESULTS

Several leukemia and lymphoma cell lines express Dll4

We asked whether leukemia/lymphoma cells express Dll4. For that, we collected protein from eight leukemia/lymphoma cell lines, namely, HL-60 (AML), 697 (B-ALL), MOLT-4 (T-ALL), HEL (erythroleukemia), JVM-13 (B-prolymphocytic leukemia), DoHH2 (B cell non-Hodgkin's lymphoma), Raji (Burkitt's lymphoma), and RCH-ACV (B-ALL). All of them were found to express Dll4 protein, and the cell lines MOLT-4 and DoHH2 were the ones expressing higher Dll4 levels (Figure 3.1A).

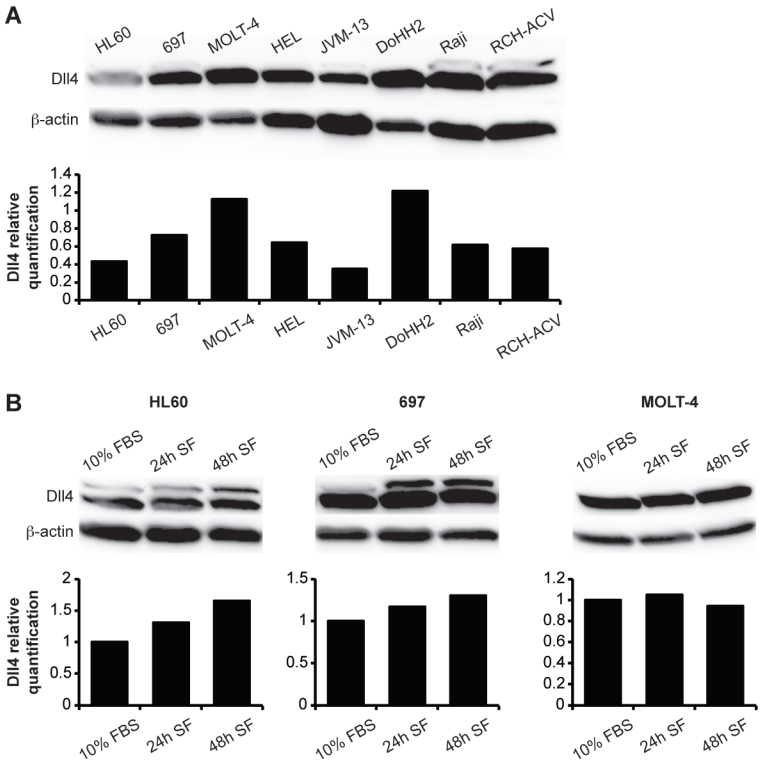


Figure 3.1. Several leukemia and lymphoma cell lines express Dll4.
A. Western blotting of Dll4 in the cell lines HL60, 697, MOLT4, HEL, JVM-13, DoHH2, Raji and RCH-ACV. Delta-like 4 quantification, relative to β-actin, shows all the cell lines express Dll4, in different quantities.
B. Western blotting of Dll4 in the cell lines HL60, 697 and MOLT4 in serum-rich (10%FBS) and serum-free (SF) conditions. Delta-like 4 quantification, relative to β-actin, shows Dll4 protein in all cell lines both in 10% FBS and SF conditions.

Interestingly, HL60, DoHH2 and Raji had been previously shown to express Dll4 [50-52]. However, MOTL-4 is reported to do not express Dll4, even though variable amounts of Dll4 were detected in primary T-ALL and B-ALL cells [53]. All of the previous reports assessed Dll4 mRNA expression by reverse transcription polymerase chain reaction (RT-PCR), but not at the protein level. We provide evidence of Dll4 protein expression by these cell lines.

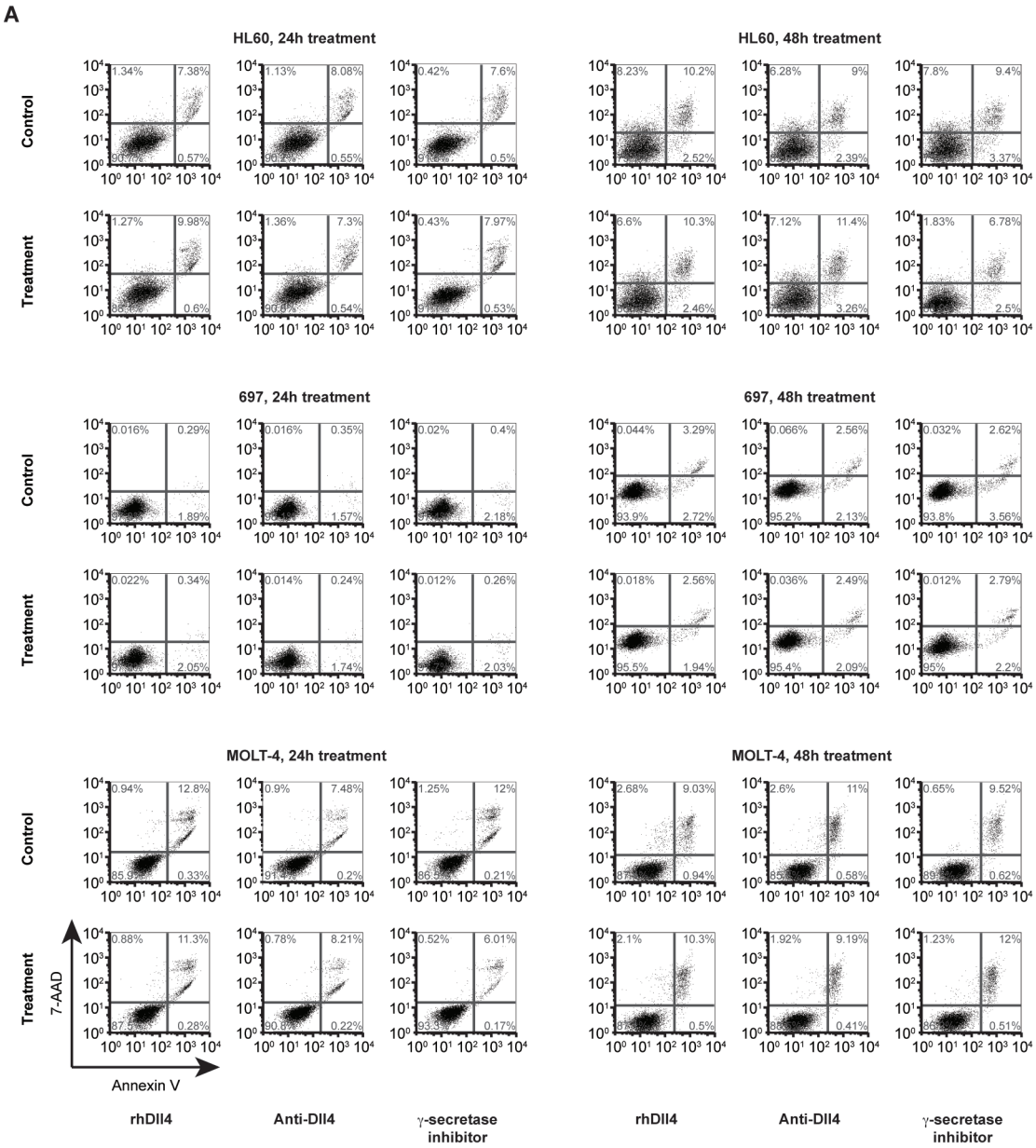
We then decided to focus in three cell lines, HL-60, 697 and MOLT-4, representatives of three different hematological malignancies, AML, B- and T-ALL. In order to assess whether Dll4 expression could be compromised in serum-free conditions, in which further experiments should be done, we measured Dll4 protein levels after 24 and 48 hours of starvation (Figure 3.1B). We found Dll4 protein is still expressed in HL-60, 697 and MOLT-4 cells after 24 and 48 hours of starvation (Figure 3.1B).

Notch signaling pathway does not affect spontaneous leukemia cell apoptosis

Given that the leukemia cell lines expressed Dll4, we asked whether Dll4-mediated Notch signaling would affect cell survival. For this, we cultured HL-60, 697 and MOLT-4 cells in serum free conditions for 24 or 48 hours, and treated them with rhDll4, anti-Dll4 or γ-secretase inhibitor, to induce or inhibit Dll4-mediated Notch signaling and to inhibit the activation of Notch receptors. As shown in figure 3.2, neither of the treatments affected any cell line (Figure 3.2).

Previous reports have shown γ-secretase inhibitors decrease the cell viability of different AML, B- and T-ALL cell lines (including HL-60 and 697), and to a much lesser extent normal B lymphocytes [51,54-56]. It is noteworthy to mention that these observations were performed using different γ-secretase inhibitors; in fact, using 10μM DAPT, these studies report results similar to the ones presented in this paper [55,56].

To our knowledge, the only study that assessed Dll4 involvement in leukemia cell survival reports a T-ALL cell line, Jurkat, to be unaffected by rhDll4 treatment [57].



We provide, for the first time, evidence that neither modulation of Dll4:Notch signaling pathway nor inhibition of Notch signaling affect leukemia cell survival.

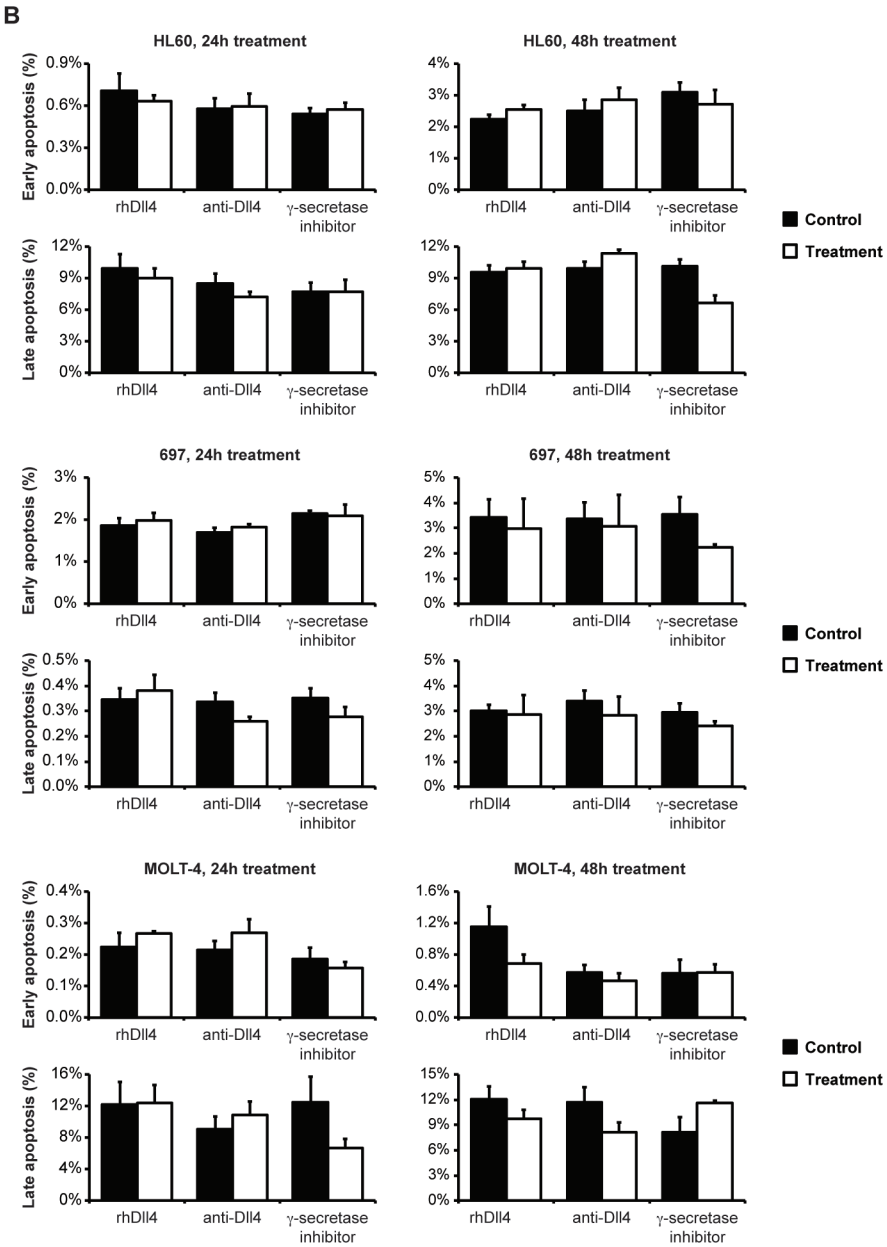
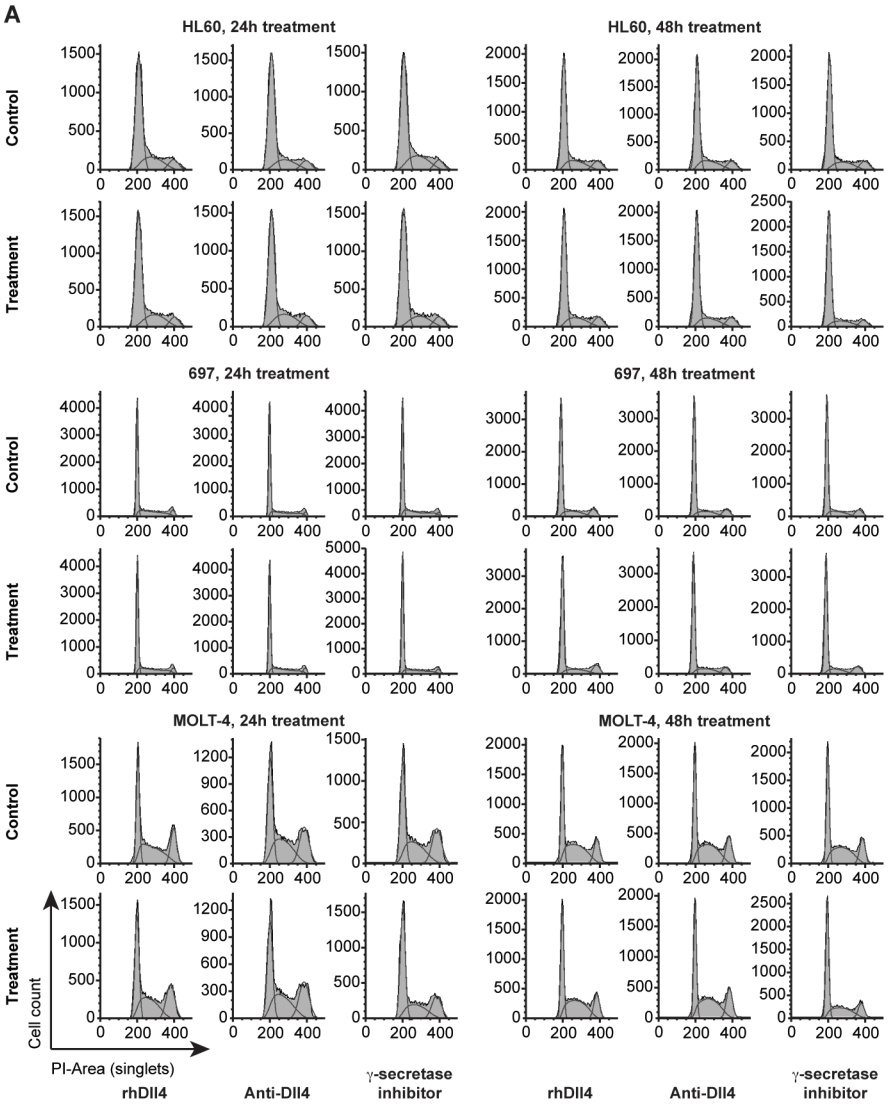


Figure 3.2. Modulation of Dll4-mediated Notch signaling and canonical Notch signaling inhibition does not affect leukemia cell apoptosis.
Flow cytometric analysis revealed that HL60, 697, and MOLT-4 survival is not directly affected by rhDll4, anti-Dll4 nor γ-secretase inhibiton.
A. Representative flow cytometry plots for leukemia cell apoptosis analysis. Data represent one of 4 replicates.
B. The percentage of leukemia cell early and late apoptosis. Data are means ± s.e.m. n=4.

Notch signaling pathway promotes leukemia cell growth arrest in a Dll4 independent manner

Several studies have previously shown that the blockade of the Notch receptor activation, through γ-secretase inhibitors, induces leukemia cell growth arrest, namely AML, Burkitt's lymphoma and several T-ALL



cell lines [3,50,51,54]. Interestingly, Dll4 blockade reduced solid leukemia growth *in vivo* (using AML and T-ALL cell lines), by affecting tumor angiogenesis and blocking Dll4:Notch3 signaling in the tumor cells [12-15]. We thus asked whether Dll4:Notch signaling could alter leukemia cell growth *in vitro*.

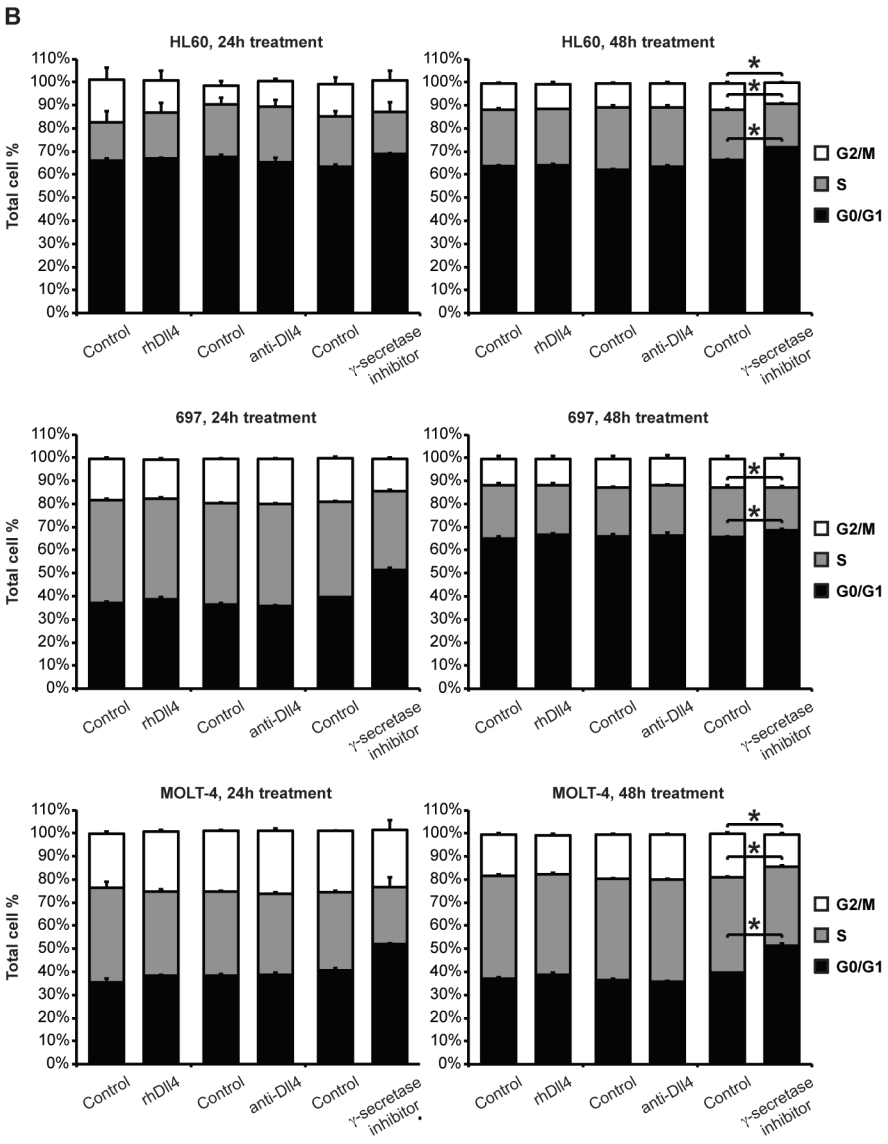


Figure 3.3. Notch signaling inhibition reduces leukemia cell proliferation, independently of Dll4.
Flow cytometric analysis of cultured HL60, 697, and MOLT-4 reveals a reduced proliferation upon γ-secretase inhibition, 48 hours treatment, but not upon rhDll4 nor anti-Dll4 treatment.
A. Representative flow cytometry plots for leukemia cell cycle analysis. Data represent one of 4 replicates.
B. The percentage of leukemia cell in G2/M, S, and G0/G1 phases of the cell cycle. Data are means ± s.e.m. n=4. s.e.m. n=4.

We treated HL-60, 697 and MOLT-4 cells with either rhDll4, anti-Dll4 or γ-secretase inhibitor. Twenty-four hours after treatment cell growth remained unaltered, however, after 48 hours of treatment, all cell lines exhibited an increased cell percentage in G_0/G_1 phase of the cell cycle,

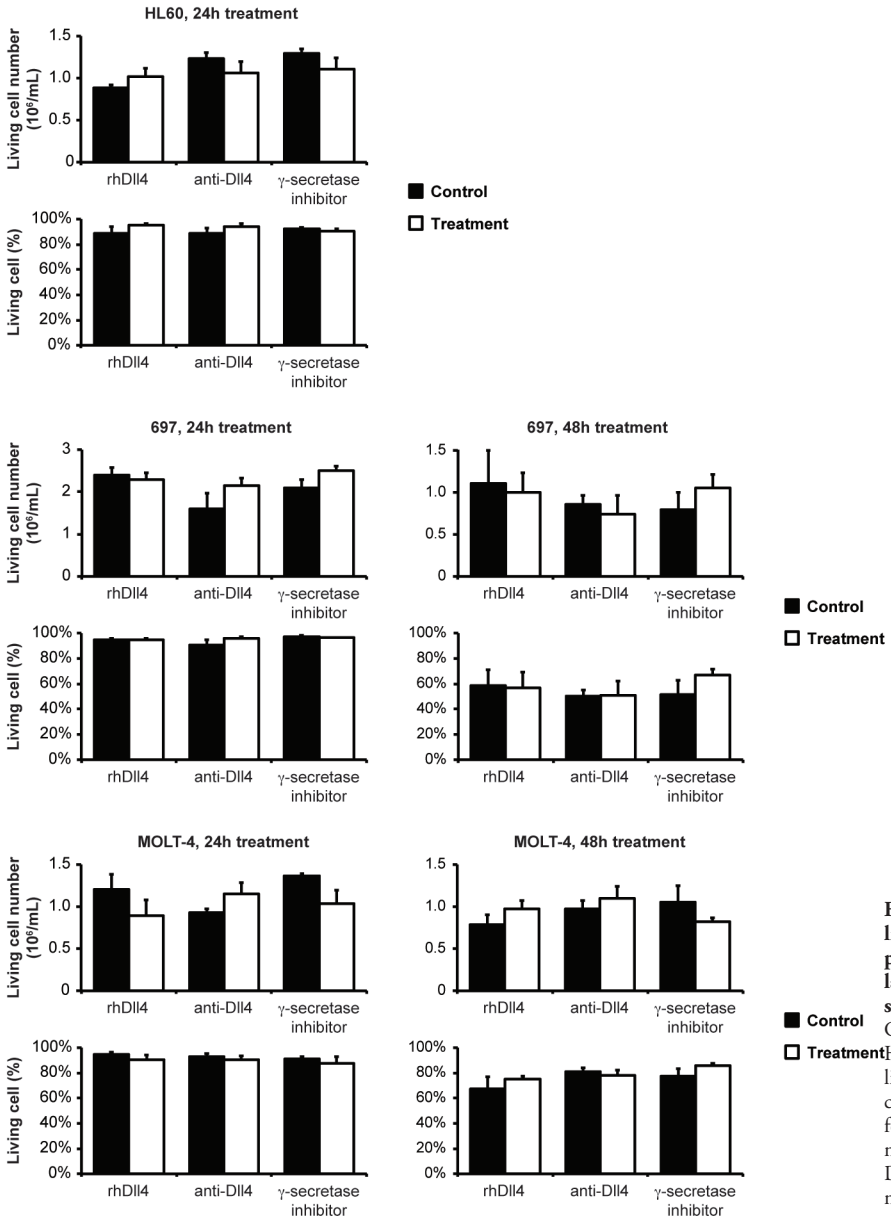


Figure 3.4. Leukemia living cell counts and percentage are not modulated by Dll4 or Notch signaling. Cell counts reveal that HL60, 697, and MOLT-4 living cell number and percentage is not directly affected by rhDll4, anti-Dll4 nor γ-secretase inhibiton. Data are means ± s.e.m. n=4.

with a decrease of cells in the S phase, and in the case of the AML and T-ALL cell lines HL-60 and MOLT-4, a reduced G_2/M cell percentage upon γ-secretase treatment (Figure 3.3). Interestingly, neither rhDll4 nor anti-Dll4 treatments significantly changed the percentage of cells in any phase of the cell cycle (Figure 3.3). Both the living cell number and percentage remained unaltered in all conditions (Figure 3.4).

DISCUSSION

The functional role of Dll4:Notch pathway in hematological tumors is poorly understood. We provide evidence that Dll4 is expressed in several leukemia/lymphoma cell lines, and that Dll4:Notch blockade or stimulation *in vitro*, up to 48 hours, does not directly affect leukemia cell survival or growth. We further show that inhibition of the Notch signaling pathway, using the γ-secretase inhibitor DAPT, does not affect leukemia cell survival but promotes leukemia cell cycle arrest.

As previously mentioned, the role of Notch signaling pathway in leukemia cell survival is not yet clear, as different γ-secretase inhibitors provide different results [51,54-56]. In fact, γ-secretase is not exclusive of the Notch signaling pathway. Besides cleaving Notch receptors [58] and ligands [59], it is also involved in other signaling molecules such as E-Cadherin [60], CD44 [61], ErbB-4 [62] and low density lipoprotein receptor-related protein [63,64]. Furthermore, there are different γ-secretase inhibitors with different chemistries: peptide isosteres (such as DAPT), azepines, and sulfonamides [65]. Therefore, different γ-secretase inhibitors may have different affinities to different transmembrane proteins, thus having different effects.

We report here that γ-secretase inhibition promotes leukemia cell cycle arrest. We show that neither Dll4:Notch signaling blockade nor stimulation affect leukemia cell cycle. Other Notch ligands may be responsible for the cell cycle arrest induced by γ-secretase inhibitors. Notably, Jagged-1-mediated Notch signaling has been shown to induce HL-60

cell cycle arrest, even though others show Jagged-1 does not affect HL-60 proliferation [51,66].

The use of anti-Dll4 antibodies for the treatment of leukemia has been previously suggested [13]. We show here that Dll4 modulation does not directly affect leukemia cell survival or proliferation.

REFERENCES

1. Kopan, R. & Ilagan, M. X. G. The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism. *Cell* 137, 216–233 (2009).

2. Ellisen, L. W. *et al.* TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* 66, 649–661 (1991).

3. Weng, A. P. *et al.* Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* 306, 269–271 (2004).

4. Zhang, J. *et al.* The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. *Nature* 481, 157–163 (2012).

5. Pear, W. S. *et al.* Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J. Exp. Med.* 183, 2283–2291 (1996).

6. Bellavia, D. *et al.* Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice. *EMBO J.* 19, 3337–3348 (2000).

7. Yan, X. Q. *et al.* A novel Notch ligand, Dll4, induces T-cell leukemia/lymphoma when overexpressed in mice by retroviral-mediated gene transfer. *Blood* 98, 3793–3799 (2001).

8. Dorsch, M. *et al.* Ectopic expression of Delta4 impairs hematopoietic development and leads to lymphoproliferative disease. *Blood* 100, 2046–2055 (2002).

9. Patel, N. S. *et al.* Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. *Cancer Research* 65, 8690–8697 (2005).

10. Noguera-Troise, I. *et al.* Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* 444, 1032–1037 (2006).

11. Ridgway, J. *et al.* Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature* 444, 1083–1087 (2006).

12. Indraccolo, S. *et al.* Cross-talk between Tumor and Endothelial Cells Involving the Notch3-Dll4 Interaction Marks Escape from Tumor Dormancy. *Cancer Research* 69, 1314–1323 (2009).

13. Indraccolo, S., Minuzzo, S., Masiero, M. & Amadori, A. Ligand-driven activation of the notch pathway in T-ALL and solid tumors: why Not(ch)? *Cell Cycle* 9, 80–85 (2010).

14. Real, C. *et al.* Bone marrow-derived endothelial progenitors expressing Delta-like 4 (Dll4) regulate tumor angiogenesis. *PLoS ONE* 6, e18323 (2011).

15. Masiero, M. *et al.* Notch3-mediated regulation of MKP-1 levels promotes survival of T acute lymphoblastic leukemia cells. *Leukemia* 25, 588–598 (2011).

16. Phng, L. K. & Gerhardt, H. Angiogenesis: a team effort coordinated by notch. *Developmental Cell* 16, 196–208 (2009).

17. A Multiple-Ascending-Dose Study of the Safety and Tolerability of REGN421(SAR153192) in Patients With Advanced Solid Malignancies - Full Text View - ClinicalTrials.gov. 1–3 (2012).

18. A Phase 1 Dose Escalation Study of OMP-21M18 in Subjects With Solid Tumors - Full Text View - ClinicalTrials.gov. 1–3 (2012).

19. Zhang, J. *et al.* Prognostic impact of δ-like ligand 4 and Notch1 in acute myeloid leukemia. *Oncol. Rep.* 28, 1503–1511 (2012).

20. Zhang, J. *et al.* Cross-talk between Leukemic and Endothelial cells Promotes Angiogenesis by VEGF Activation of the Notch/Dll4 Pathway. *Carcinogenesis* (2012). doi:10.1093/carcin/bgs386

21. Hoey, T. *et al.* DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency. *Cell Stem Cell* 5, 168–177 (2009).

22. Yen, W.-C. *et al.* Anti-DLL4 Has Broad Spectrum Activity in Pancreatic Cancer Dependent on Targeting DLL4-Notch Signaling in Both Tumor and Vasculature Cells. *Clinical Cancer Research* 18, 5374–5386 (2012).

23. Fadok, V. A. *et al.* Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* 148, 2207–2216 (1992).

24. Martin, S. J. *et al.* Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* 182, 1545–1556 (1995).

25. Verhoven, B., Schlegel, R. A. & Williamson, P. Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J. Exp. Med.* 182, 1597–1601 (1995).

26. Tait, J. F., Gibson, D. & Fujikawa, K. Phospholipid binding properties of human placental anticoagulant protein-I, a member of the lipocortin family. *J. Biol. Chem.* 264, 7944–7949 (1989).

27. Andree, H. A. *et al.* Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. *J. Biol. Chem.* 265, 4923–4928 (1990).

28. Koopman, G. *et al.* Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 84, 1415–1420 (1994).

29. Vermes, I., Haanen, C., Steffens-Nakken, H. & Reutelingsperger, C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods* 184, 39–51 (1995).

30. Homburg, C. H. *et al.* Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis in vitro. *Blood* 85, 532–540 (1995).

31. Schmid, I., Krall, W. J., Uittenbogaart, C. H., Braun, J. & Giorgi, J. V. Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. *Cytometry* 13, 204–208 (1992).

32. Klinakis, A. *et al.* A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia. *Nature* 473, 230–233 (2011).

33. Krishan, A. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. *The Journal of Cell Biology* 66, 188–193 (1975).

34. Fried, J., Perez, A. G. & Clarkson, B. D. Flow cytofluorometric analysis of cell cycle distributions using propidium iodide. Properties of the method and mathematical analysis of the data. *The Journal of Cell Biology* 71, 172–181 (1976).

35. Collins, S. J., Gallo, R. C. & Gallagher, R. E. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature* 270, 347–349 (1977).

36. Findley, H. W., Cooper, M. D., Kim, T. H., Alvarado, C. & Ragab, A. H. Two new acute lymphoblastic leukemia cell lines with early B-cell phenotypes. *Blood* 60, 1305–1309 (1982).

37. Minowada, J., Onuma, T. & Moore, G. E. Rosette-forming human lymphoid cell lines. I. Establishment and evidence for origin of thymus-derived lymphocytes. *J. Natl. Cancer Inst.* 49, 891–895 (1972).

38. Martin, P. & Papayannopoulou, T. HEL cells:

a new human erythroleukemia cell line with spontaneous and induced globin expression. *Science* 216, 1233–1235 (1982).

39. Melo, J. V., Foroni, L., Brito-Babapulle, V., Luzzatto, L. & Catovsky, D. The establishment of cell lines from chronic B cell leukaemias: evidence of leukaemic origin by karyotypic abnormalities and Ig gene rearrangement. *Clin. Exp. Immunol.* 73, 23–28 (1988).

40. Kluin-Nelemans, H. C. *et al.* A new non-Hodgkin's B-cell line (DoHH2) with a chromosomal translocation t(14;18)(q32;q21). *Leukemia* 5, 221–224 (1991).

41. Pulvertaft, J. V. Cytology of Burkitt's tumour (african lymphoma). *Lancet* 1, 238–240 (1964).

42. Jack, I. *et al.* RCH-ACV: a lymphoblastic leukemia cell line with chromosome translocation 1;19 and trisomy 8. *Cancer Genet. Cytogenet.* 19, 261–269 (1986).

43. Williams, C. K., Li, J.-L., Murga, M., Harris, A. L. & Tosato, G. Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. *Blood* 107, 931–939 (2006).

44. Caiado, F., Real, C., Carvalho, T. & Dias, S. Notch pathway modulation on bone marrow-derived vascular precursor cells regulates their angiogenic and wound healing potential. *PLoS ONE* 3, e3752 (2008).

45. Caolo, V. *et al.* Feed-forward signaling by membrane-bound ligand receptor circuit: the case of Notch Delta-like 4 ligand in endothelial cells. *Journal of Biological Chemistry* 285, 40681–40689 (2010).

46. Sunamura, M. & Yagita, H. Neutralizing monoclonal antibody against human Dll4. (2008).

47. Sunamura, M. & Yagita, H. Anti-Dll4 binding protein. *UK Patent Application* 1–85 (2008).

48. Dovey, H. F. *et al.* Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. *J. Neurochem.* 76, 173–181 (2001).

49. Benedito, R. *et al.* Notch-dependent VEGFR3 upregulation allows angiogenesis without VEGF-VEGFR2 signalling. *Nature* 484, 110–114 (2012).

50. Tohda, S. *et al.* Establishment of a novel B-cell lymphoma cell line with suppressed growth by gamma-secretase inhibitors. *Leuk. Res.* 30, 1385–1390 (2006).

51. Li, G.-H. *et al.* Notch signaling maintains proliferation and survival of the HL60 human promyelocytic leukemia cell line and promotes the phosphorylation of the Rb protein. *Mol. Cell. Biochem.* 340, 7–14 (2010).

52. Ramakrishnan, V. *et al.* MRK003, a γ-secretase inhibitor exhibits promising in vitro pre-clinical activity in multiple myeloma and non-Hodgkin's lymphoma. *Leukemia* 26, 340–348 (2012).

53. Chiaramonte, R. *et al.* A wide role for NOTCH1 signaling in acute leukemia. *Cancer Lett.* 219, 113–120 (2005).

54. Lewis, H. D. *et al.* Apoptosis in T cell acute lymphoblastic leukemia cells after cell cycle arrest induced by pharmacological inhibition of notch signaling. *Chem. Biol.* 14, 209–219 (2007).

55. Kogoshi, H., Sato, T., Koyama, T., Nara, N. & Tohda, S. Gamma-secretase inhibitors suppress the growth of leukemia and lymphoma cells. *Oncol. Rep.* 18, 77–80 (2007).

56. Meng, X. *et al.* GSI-I (Z-LLNle-CHO) inhibits γ-secretase and the proteosome to trigger cell death in precursor-B acute lymphoblastic leukemia. *Leukemia* 25, 1135–1146 (2011).

57. Hajdu, M., Kopper, L. & Sebestyén, A. Notch-regulation upon Dll4-stimulation of TGFb-induced apoptosis and gene expression in human B-cell non-Hodgkin lymphomas. *Scand. J. Immunol.* 71, 29–37 (2010).

58. De Strooper, B. *et al.* A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature* 398, 518–522 (1999).

59. LaVoie, M. J. & Selkoe, D. J. The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. *J. Biol. Chem.* 278, 34427–34437 (2003).

60. Marambaud, P. *et al.* A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. *EMBO J.* 21, 1948–1956 (2002).

61. Murakami, D. *et al.* Presenilin-dependent gamma-secretase activity mediates the intramembranous cleavage of CD44. *Oncogene* 22, 1511–1516 (2003).

62. Ni, C. Y., Murphy, M. P., Golde, T. E. & Carpenter, G. gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science* 294, 2179–2181 (2001).

63. May, P., Reddy, Y. K. & Herz, J. Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain. *J. Biol. Chem.* 277, 18736–18743 (2002).

64. Kopan, R. & Ilagan, M. X. G. Gamma-secretase: proteasome of the membrane? *Nat Rev Mol Cell Biol* 5, 499–504 (2004).

65. Olson, R. E. & Albright, C. F. Recent progress in the medicinal chemistry of gamma-secretase inhibitors. *Curr Top Med Chem* 8, 17–33 (2008).

66. Zweidler-McKay, P. A. *et al.* Notch signaling is a potent inducer of growth arrest and apoptosis in a wide range of B-cell malignancies. *Blood* 106, 3898–3906 (2005).

67. Tsiftsoglou, A. S., Pappas, I. S. & Vizirianakis, I. S. Mechanisms involved in the induced differentiation of leukemia cells. *Pharmacol. Ther.* 100, 257–290 (2003).

68. Koch, U. *et al.* Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *Journal of Experimental Medicine* 205, 2515–2523 (2008).

69. Hozumi, K. *et al.* Delta-like 4 is indispensable in thymic environment specific for T cell development. *Journal of Experimental Medicine* 205, 2507–2513 (2008).

70. Mohtashami, M. *et al.* Direct comparison of Dll1- and Dll4-mediated Notch activation levels shows differential lymphomyeloid lineage commitment outcomes. *The Journal of Immunology* 185, 867–876 (2010).

71. Remédio, L. *et al.* Context- and cell-dependent effects of delta-like 4 targeting in the bone marrow microenvironment. *PLoS ONE* 7, e52450 (2012).

72. Sarmento, L. M. *et al.* Notch1 modulates timing of G1-S progression by inducing SKP2 transcription and p27 Kip1 degradation. *J. Exp. Med.* 202, 157–168 (2005).

4

CHRONIC INTERMITTENT HYPOXIA AFFECTS HEMATOPOIESIS AND MODULATES THE BONE MARROW MICROENVIRONMENT

In preparation. Remédio L., Martin I., Matias I., Diogo L., Monteiro E. C. and Dias S.

CONTENTS

ABSTRACT **104**

INTRODUCTION **105**

METHODS **107**

Animals and experimental design 107

Sample collection 108

Pimonidazole Staining 108

Histological and immunohistochemical analysis 108

Reverse transcriptase PCR (RT–PCR) 109

RESULTS **110**

Chronic intermittent hypoxia modulates hematopoiesis 110

Chronic intermittent hypoxia interferes with the BM vascular niche 112

Chronic intermittent hypoxia modulates bone marrow "angiocrine" gene expression 116

DISCUSSION **118**

REFERENCES **121**

ABSTRACT

The role of environmental hypoxia in hematopoiesis is not completely understood, and the way it may affect the BM microenvironment is unknown. This study focused on the role of chronic intermittent hypoxia (CIH), a murine model for obstructive sleep apnea, in the modulation of hematopoiesis and the BM microenvironment. Our preliminary results suggest that CIH followed by normoxia increases erythropoiesis, circulating monocyte counts and thrombocytosis. The BM hypoxia is apparently unaltered in the CIH-subjected animals, as observed by pimonidazole staining. However, we observed an increase in the number of $CD105^+$ BM vessels, and a decrease in VE-$Cadherin^+$ and SMA^+ vessels, without changes in vWF^+ vascular density. Our preliminary data also suggests there is a decrease in the total number of BM megakaryocytes, and an increase of BM megakaryocyte apoptosis in CIH-subjected animals. We further investigated the "angiocrine" gene expression in the BM, and our preliminary results suggest an increase of IL-6, Dll1, CSF2, CSF3, Smad3 and THPO and a decrease of IGF1, IGFbp3, IGFbp5, Angpt1, SCF and N-Cadherin expression. Together, our preliminary data suggest CIH followed by normoxia may modulate the BM microenvironment, perturbing hematopoiesis possibly through altered "angiocrine" gene expression. This study may clarify the role of CIH in BM hematopoiesis.

INTRODUCTION

Hematopoiesis has long been known to be affected by environmental hypoxia [1,2]. Despite the numerous reports relating hypoxia with hematopoietic modulation [1,3-25], and the great attention currently given to hypoxia-inducible factor (HIF) in hematopoiesis and bone marrow (BM) microenvironment [26-29], the role of environmental hypoxia in the BM microenvironment is still largely unknown. We report the hematopoietic and BM vascular compartment modulation during a clinically relevant hypoxia approach, obstructive sleep apnea, in which the patients are subjected to chronic intermittent hypoxia (CIH) during their sleep period [30,31].

Hematopoiesis occurs in the adult mammalian BM, where hematopoietic stem cells (HSCs) are believed to reside in hypoxic niches [26,32-37]. These HSCs are currently thought to localize near the endosteal (bone) area of the BM, richly vascularized but poorly perfused [34,38]. The role of oxygen tension (pO_2) in hematopoiesis has been assessed both *in vitro* and *in vivo*. The *in vitro* studies typically compare cultures in 1 or 5% (7mmHg or 38mmHg) O_2 (hypoxia) to cultures in 20-21% (150mmHg) O_2 (normoxia) [12,15,17,18,20,25,39]. However, this pO_2 does not reflect the BM pO_2, which is 13.9-27.7mmHg in dogs and 54.9mmHg in humans – about one third of the value used as normoxia [40,41]. The *in vivo* studies were performed either by environmental hypoxia, or by modulating the master regulator of oxygen homeostasis hypoxia inducible factor (HIF)-α levels either pharmacologically or genetically [1,3,6-9,11,13,14,16,26,29,42]. HIF-α is degraded by the proteasome in most cell types in normoxia [43]. However, HIF-α was shown to be stable also in normoxic conditions in both monocytes and leukemia cell lines [44,45]. Therefore, and because specific modulation of pO_2 in the BM is still technically unachievable, we consider that the best available tool to modulate pO_2 in the BM microenvironment is environmental hypoxia. In fact, the fraction of inspired oxygen is correlated with arterial pO_2 and BM pO_2 during acute modulations of atmospheric O_2 [46].

Hematopoietic responses to environmental hypoxia have been extensively studied in the past, however, the experimental designs were highly variable, typically not corresponding to any clinically relevant evaluation, and technical restraints limited the scientific knowledge extracted from these studies [1,3,6-9,11,13,14,16,42]. The most consistent hematopoietic modulation triggered by hypoxia is the promotion of erythropoiesis [2], however, the modulation of other hematopoietic parameters is still largely unknown, being highly variable according to the hypoxia exposure.

The modulation of the BM microenvironment after hypoxia exposure is also unknown. We have focused on the vascular compartment of the BM, because it mediates the differentiation and proliferation of hematopoietic cells, as well as its egress from the BM [47]. Furthermore, one of the most striking effects of hypoxia is the promotion of angiogenesis, for which the most preeminent mechanism is the HIF-mediated vascular endothelial factor (VEGF) increased expression [48].

We assessed the role of environmental hypoxia using a clinically relevant system, which consists in exposing the experimental subjects in CIH for 30-35 days, as a model of obstructive sleep apnea syndrome [49]. The clinical hematological aspects of obstructive sleep apnea are still largely unknown, with several studies assessing mainly platelet activation, but not complete blood count (CBC) [30]. Furthermore, these studies typically compare groups of patients with different levels of the disease [50], or treated versus untreated patients [51], but not healthy controls versus patients [30].

We collected the data on changes in hematopoietic parameters using easily accessible tools for the clinic, such as CBC. To observe the hypoxic areas in the BM, we used a misonidazole-based compound (Hypoxyprobe), which forms adducts with thiol groups of proteins, peptides and aminoacids specifically in hypoxic cells (pO_2<10mmHg) [52-54]. We further explored the possible modulation of hematopoiesis by the

BM vascular compartment by assessing "angiocrine" genes expression, a set of genes considered to be the effectors for the instructive role of the BM vascular niche [55].

Our preliminary data suggest that CIH may promote erythropoiesis, increase blood monocyte and platelet count and reduce platelet size. Furthermore, CIH may modify the BM vascular compartment, by increasing $CD105^+$ vessels and reducing VE-$Cadherin^+$ and smooth muscle actin $(SMA)^+$ vessels, without modifying von Willebrand factor $(vWF)^+$ vessel coverage, and decreasing BM vWF^+ megakaryocyte numbers and inducing megakaryocyte apoptosis. Our preliminary results further suggest these effects of CIH may be a result of "angiocrine" genes modulation.

METHODS

Animals and experimental design

The following animal experiments were performed with the approval of the Institutional Ethics Committee of the Faculdade de Ciências Médicas (Nova University, Lisbon) and the national authorities (DGV) for animal care and use in research.

Four male Wistar rats, aged 3 months and weighting 400-500g, were housed in pexiglas boxes, 1 per box, with *ad libitum* access to food and water. The cages were kept in a medium A-chamber (A-30274-P), in an atmosphere controlled using an OxyCycler AT series (all from Biospherix Ltd, NY, USA), by electronically regulated solenoid switches in a three-channel gas mixer, which gradually lowered oxygen in the chamber over 90 seconds from 21% to 5% O_2 and increased the CO_2 content from 0% to 5%. The O_2 and CO_2 were purchased as regular gas bottles, while N_2 was generated from the air by pressure swing adsorption technology using a high output nitrogen generator (Nitrogen 15 Plus, PSA Technology, Sysadvance, Maia, Portugal) (Figure 4.1A). Rats were exposed to 20 chronic intermittent hypoxia cycles per hour for 8 hours a day

during their sleep period for 32 days, and analyzed 3 days after that. The rats subjected to hypoxia had a lower body weight than controls (Figure 4.1B), as has been previously documented in different types of hypoxia [56-58], despite the fact that patients with obstructive sleep apnea have higher body mass index [59].

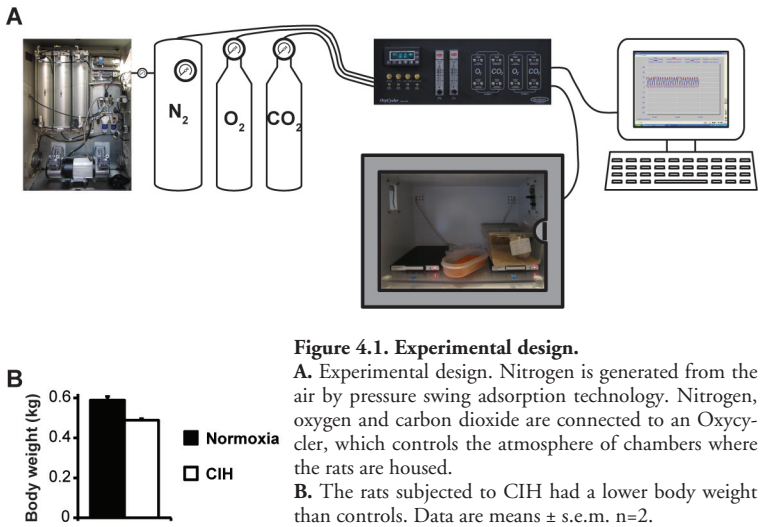


Figure 4.1. Experimental design.
A. Experimental design. Nitrogen is generated from the air by pressure swing adsorption technology. Nitrogen, oxygen and carbon dioxide are connected to an Oxycycler, which controls the atmosphere of chambers where the rats are housed.
B. The rats subjected to CIH had a lower body weight than controls. Data are means ± s.e.m. n=2.

Sample collection

Peripheral blood was collected from the heart in EDTA-coated tubes (Multivette 600, Sarstedt, Nümbrecht, Germany) and CBC were performed.

Pimonidazole Staining

Thirty-five days after the initiation of the experiment, 60mg/kg pimonidazole hydrochloride (Hypoxyprobe, Inc, Burlington, USA) was administered intravenously. Two hours later, rats were euthanized using 60mg/kg sodium pentobarbital IV (Eutasil, Ceva Santé Animale, Libourne, France) and transcardially perfused with phosphate buffered saline (PBS).

Histological and immunohistochemical analysis

Femurs were formalin-fixed, decalcified with formic acid for three days, and processed for routine histopathology.

Immunohistochemistry for the antigens indicated on Table 4.1 was performed on 3μm slices, at 3 distinct levels for each bone/rat (40μm distance). Sections were incubated with primary antibody at room temperature for 1h, immunostaining proceeded according to the visualization system manufacturer's instructions and counterstained with Mayer's hematoxylin.

Table 4.1. Antibodies list.

Antigen	Antigen Retrieval	Dilution	Clone	Brand
CD105 (Endoglin)	HIAR	1:150	Polyclonal	R&D AF1320
SMA	PIER	1:200	M0851	DAKO M0851
vWF	PIER	1:300	Polyclonal	DAKO A0082
VE-Cadherin	PIER	1:150	Polyclonal	R&D AF1002
Pimonidazole	HIAR	1:200	Polyclonal	Hypoxyprobe PAb2627AP
Anti-Goat, peroxidase	Immunohistochemistry	ready-to-use	—	VectorLabs MP-7405
Anti-Mouse, peroxidase	Immunohistochemistry	ready-to-use	—	DAKO K4007
Anti-Rabbit, peroxidase	Immunohistochemistry	ready-to-use	—	DAKO K4011

Reverse transcriptase PCR (RT–PCR)

For *in vivo* assessments, total BM from control or CIH rats was flushed off in PBS, centrifuged 1200rpm 5min, and collected to TRIzol Reagent (Invitrogen, Carlsbad, CA).

RNA was extracted according to the manufacturer's instructions. cDNA was produced with SuperScript II (Invitrogen, Carlsbad, CA) by using random-sequence hexamer primers (Roche Applied Science, Indianapolis, IN). Real-time PCR was performed with Power SYBR Green PCR Master Mix in 7900HT Fast Real-Time PCR System (both from Applied Biosystems, Foster City, CA). Amplification of hypoxanthine guanine phosphoribosyl transferase (HPRT) was used for sample normalization. Primer sequences are as described on Table 4.2.

Table 4.2. Primers list.

mHPRT	F	GACCGCTTTTCCCGCGAGCC
	R	TCACGACGCTGGGACTGAGGG
Angpt1	F	TGATGCCTGTGGCCCTTCCA
	R	CATGGTTTTGCCCCGCAGTGT
Angpt2	F	TGTCCGGCGAGGAGTCCAAC
	R	GATTTTGCCCGCCGTGCCTG
CD31	F	TGGCTTGAGTGGGCGGATGG
	R	AGCCGGGTGGCTGAGGGAAG
CSF1	F	GCCACCGAGAGGCTACAGGAA
	R	TTTGGACACAGGCCTCGTTCTGTT
CSF2	F	GGTCTACGGGGCAACCTCACC
	R	AGTTTCCGGGGTTGGAGGGCA
CSF3	F	CCTCGGGGTGGCCCCTACTG
	R	CCCGACGCTGGAAGGCAGAA
Dll1	F	TCTCCTGACGACCTCGCAACA
	R	GGTGCCTCTGTGTGGTCAGGC
Dll4	F	CTGGCCGGGAACCTTCTCACTC
	R	TCTCTGGCCGCAGGTCGTCTC
FGF1	F	AGGGACAGGAGCGACCAGCA
	R	TACACTTCGCCCGCGCTTTCC
Flt3L	F	AGCTCTGAAGCCCTGTATCGGGA
	R	ACTGCACCTCCAGGCACCGA
Hey2	F	CCCTTGCGAGGAGACGACCT
	R	GCTCCCCACGTCGATGGTCT
IGF1	F	CTTTGCGGGGCTGAGCTGGT
	R	AGCCCCTTGGTCCACACACGAA
IGFbp3	F	AAGGCGCTGCTGAATGGCCG
	R	GCTGGGAGGGGAGGTAGGCA
IGFbp5	F	ACCTGCCCAACTGTGACCGC
	R	GGCCACGAGAAGGCTTGCACT
IL-3	F	TGATGCTCTTCCACCAGGGACT
	R	AGTCCTGCAATCCAACGTCCTGA
IL-6	F	CTCTCCGCAAGAGACTTCCAGC
	R	AGGGAAGGCAGTGGCTGTCAA
Jagged1	F	GGAAGGCTGGATGGGTCCTGA
	R	TGCAGGAGCCATGCTTGGGA
Jagged2	F	CGGGCCTCGTCGTCATTCCCT
	R	CAGGCCTCCACGATGAGGGTGA
N-Cadherin	F	TCTGCACCAGGTTTGGAATGGGT
	R	ACATACGTCCCAGGCTTTGATCCC
SCF	F	ACAAAACTGGTGGCGAATCTTCCAA
	R	TCCCGGCGACATAGTTGAGGGT
Smad2	F	TGTGCAGAGCCCCAACTGTAACCA
	R	GGATTTTGCACACTGTCGCGGG
Smad3	F	AGGCCATCACCACGCAGAACG
	R	AGCCGGCCATCCAGTGACCT
TGFβ1	F	AGCCCGAGGCGGACTACTAC
	R	TGCGTTGTTGCGGTCCACCATT
THPO	F	TGTCCCCACCCCACTCTGTGC
	R	GTGTGGGGCCTCTCCCCTGA
VCAM1	F	CGGAGCCTCAACGGTACTTTGG
	R	GCGAGCGTTTTGTATTCAGGGGA

RESULTS

Chronic intermittent hypoxia modulates hematopoiesis

We started our studies by asking whether CIH had any effect on hematopoiesis. For this purpose, rats were exposed to 20 chronic intermittent hypoxia cycles per hour (cycles of 21% – 5% over 90 seconds) for 8 hours a day during their sleep period for 32 days, and analyzed after 3 days at room air. The posthypoxic period before the analysis allowed us to observe the persistent changes in hematopoiesis the underlying changes occurring in the BM microenvironment.

We found CIH may increase erythrocyte counts, hemoglobin concentration, and hematocrit (Figure 4.2A). Previous studies show both acute and chronic hypoxia, as well as CIH, promote erythropoiesis [1,3,6,7,16,42]. This is however dependent upon the hypoxia posology, as in other studies both chronic hypoxia and chronic intermittent asphyxia do not modify circulating erythrocyte parameters [4,57]. Interestingly, clinical data of obstructive sleep apnea patients consistently show a slight increase in hematocrit, however, the data regarding erythrocyte count and hemoglobin levels is inconsistent and lacks healthy controls [30,50].

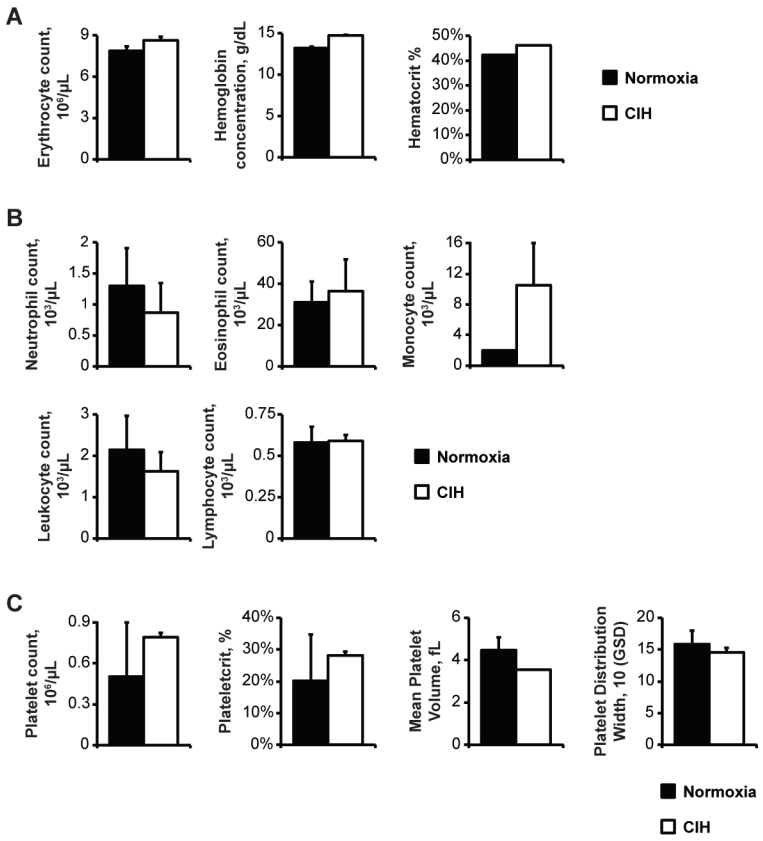


Figure 4.2. Chronic intermittent hypoxia modulates hematopoiesis.
A. Erythrocyte, hemoglobin and hematocrit quantifications were assessed by peripheral blood cell blood counts. Chronic intermittent hypoxia may promote erythropoiesis.
B. Neutrophil, eosinophil, monocyte, leukocyte and lymphocyte quantifications were assessed by peripheral blood cell blood counts. Chronic intermittent hypoxia may increase circulating monocyte numbers.
C. Platelet, plateletcrit, mean platelet volume and platelet distribution width quantifications were assessed by peripheral blood cell blood counts. Chronic intermittent hypoxia may result in thrombocytosis.
Data are means ± s.e.m. n=2.

Leukocyte, lymphocyte and granulocyte (neutrophil and eosinophil) counts are apparently unaffected by CIH; however, the data collected thus far seems to point towards an increase in monocyte count in the rats subjected to CIH (Figure 4.2B). Interestingly, acute and chronic hypoxia were reported to increase circulating granulocyte numbers [11,60], and both *in vitro* hypoxic myeloid differentiation

and *in vivo* pharmacologic stabilization of HIF-1α increase the number of BM myeloid progenitors [12,18,29].

Our preliminary data suggest that CIH may increase platelet number and plateletcrit, and reduce the mean platelet volume without affecting the platelet distribution width (Figure 4.2C). Both acute hypoxia (3 hours) and short-term chronic hypoxia (2-4 days) were reported to promote thrombocytosis (increase of platelet counts) [8,9,24,61], whereas long-term chronic hypoxia (6-14 days) promoted thrombocytopenia (decrease of platelet counts) [8,9,11,24]. On the other hand, both acute hypoxia (6 hours) and chronic intermittent asphyxia do not change platelet count but increases platelet aggregation [24,57]. It thus seem like the platelet kinetics is highly dependent upon the hypoxia posology. Chronic hypoxia, but not acute hypoxia, was shown to promote thrombocytosis with a reduction of platelet size [24], similar to what our data suggest. Interestingly, clinical studies reveal that platelet counts do not vary between different severity types of obstructive sleep apnea, and both mean platelet volume and platelet distribution width (indicators of platelet activation) are higher in more severe types of obstructive sleep apnea [50,59,62-64]. Our data thus suggest that the CIH model for obstructive sleep apnea may not mimic the disease, as the mean platelet volume seems to decrease and the platelet count and plateletcrit seem to be modulated.

Together, these preliminary data suggest that CIH followed by a normoxia period increases erythropoiesis and promotes monocytosis and thrombocytosis.

Chronic intermittent hypoxia interferes with the BM vascular niche

Next, we investigated whether CIH might interfere with (and perturb) the BM microenvironment. Our preliminary results suggest CIH does not affect BM cellularity (Figure 4.3). Previous reports show that acute hypoxia does not affect BM cellularity, but chronic hypoxia results in a

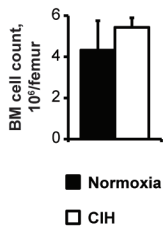


Figure 4.3. Bone marrow cell count. Total BM cell count suggests CIH does not modify BM cellularity. Data are means ± s.e.m. n=2.

hypercellular BM [6,7].

We identified the hypoxic areas in the BM using pimonidazole staining. As shown in figure 4.4A, the hypoxic BM areas seem to be equivalent between the experimental groups (Figure 4.4A). As previously mentioned, the fraction of inspired oxygen is correlated with arterial pO_2 and BM pO_2 during acute modulations (time intervals of minutes) of atmospheric O_2 [46]. Therefore, the BM of the CIH-subjected animals may not be more hypoxic either because the possible increase of erythrocytes in circulation promotes a better oxygenation in the BM, or the normoxia period subsequent to CIH was sufficient to reduce the hypoxic areas in the BM, or both.

Then we characterized the BM vessel phenotype. We used four different vascular markers to characterize the effects of CIH in the BM: CD105, VE-Cadherin and vWF antibodies, widely used markers for BM ECs [65-68] and SMA antibody, which marks pericytes both in arteries and capillaries [1,2,69-71] (Figure 4.4B). Our results suggest CIH does not affect the vWF$^+$ vessel number, but increases CD105$^+$ and decreases VE-Cadherin$^+$ and SMA$^+$ vessel density in the BM (Figure 4.4C). Several reports show hypoxia is a potent stimulator of angiogenesis [56,72-85]. Specifically, under hypoxic conditions endothelial cells upregulate CD105 expression thereby preventing apoptosis [86,87]. Furthermore, CD105 promotes endothelial cell proliferation, which may explain the increased CD105$^+$ vessel coverage [88]. Interestingly, we have previously shown CD105 stains roughly all vessels in the BM [89]. Therefore, these results may indicate an increased BM angiogenesis upon CIH.

Concomitantly, VE-Cadherin expression seems to be reduced by CIH. Interestingly, even though *in vitro* culture of BM cells in hypoxia has been shown to induce the mRNA expression of VE-Cadherin [90], pulmonary endothelial cells exposed to hypoxia *in vitro* do not modify VE-Cadherin protein levels but VE-Cadherin is dispersed from intercellular junctions, thereby permeabilizing the endothelial cell layer [91]. Our data

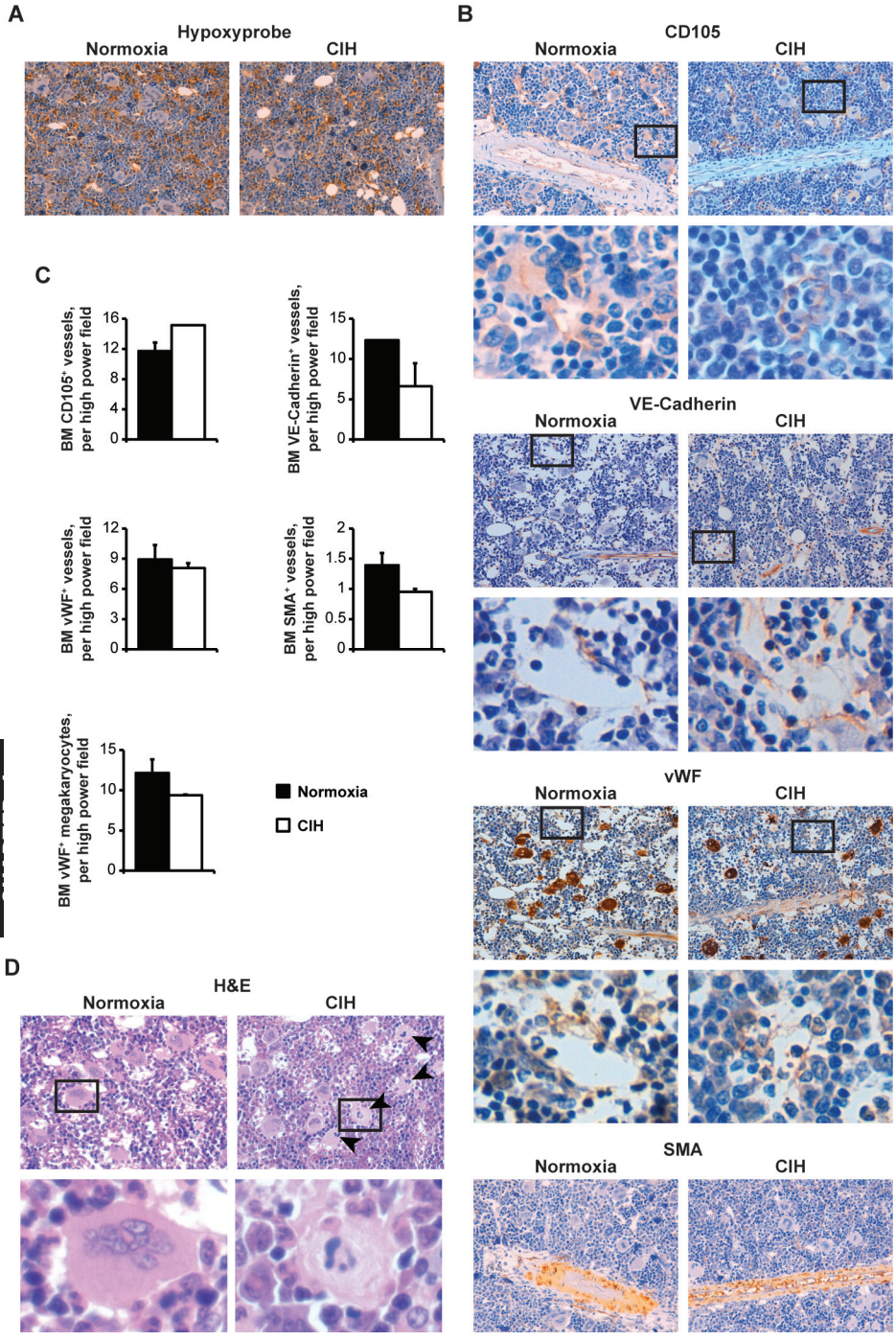


Figure 4.4. Chronic intermittent hypoxia interferes with the BM vascular niche.
A. Pimonidazole was injected 2 hours prior to sacrifice in all rats. Femur sections were stained with an anti-pimonidazole monoclonal antibody and counterstained with Mayer's haemalum (400x, Leica DMD 108).
B. Immunohistochemistry for CD105, VE-Cadherin, vWF and SMA counterstained with Mayer's haemalum (400x, Leica DMD 108; amplifications correspond to 2000x).
C. CD105, VE-Cadherin, vWF and SMA-positive vessel count, and vWF-positive megakaryocyte count, per high power field (400x, Leica DMD 108; amplifications correspond to 2000x), suggest CIH increases $CD105^+$ vessels, does not affect vWF^+ vessel numbers and reduces $VE\text{-}Cadherin^+$ vessels, SMA^+ vessels and total number of megakaryocyte count.
D. Histology of the BM evidences CIH may promote dysmegakaryopoiesis due to increased apoptosis (arrowheads); hematoxilin-eosin staining (400x, Leica DMD 108; amplifications correspond to 2000x).
Data are means ± s.e.m. n=2.

also suggest a reduced SMA^+ pericyte coverage in BM vessels. Previous reports show that hypoxia recruits pericytes to newly formed vessel sprouts, and induces pericyte proliferation [48,92-95]. In fact, most systems in which hypoxia is studied, such as lung, liver and kidney, SMA is upregulated; however, heart fibroblasts downregulate SMA expression upon hypoxia [92,96-99]. Therefore, the effect of hypoxia in different systems and vessel types is different. In fact, the data herein presented suggest that in the BM CIH reduces vessel pericyte coverage.

We also counted the number of megakaryocytes, which are part of the BM "vascular niche" [26-29,47], by using vWF as a marker [30,31,100] (Figure 4.4B). Our data suggest CIH decreases BM megakaryocyte total number (Figure 4.4C). It was previously shown that both acute and chronic hypoxia (4 days) do not affect megakaryocyte numbers, but increase the number of mature, higher ploidy megakaryocytes and megakaryocyte size [14,24,26,32-37]. An oscillatory modulation of BM megakaryocyte numbers was also previously reported, evidenced by a transient decrease only between days 6 and 10 of chronic hypoxia [13,34,38].

The pathological analysis of the BM histologic sections evidenced CIH may promote dysmegakaryopoiesis due to increased apoptosis (Figure 4.4D). Interestingly, *in vitro* studies show that hypoxia delays the maturation of megakaryocytes, reducing high ploidy, proplatelet-containing and apoptotic megakaryocytes [12,15,17,18,20,25,39]. It should be noted that BM pO_2 probably increased during the normoxic period the rats were subjected to prior to the subsequent analysis [40,41,46], thus possibly accelerating megakaryocyte maturation [1,3,6-9,11,13,14,16,20,26,29,42]. The terminal megakaryocyte differentiation stages involve apoptosis [43,101]. Thus, if more definitive results confirm our preliminary data, the increased BM megakaryocyte apopotosis, which may be related to the decrease of megakaryocyte total number, may be due to accelerated megakaryocyte maturation, and may also explain a possible thrombocytosis. Furthermore, megakaryocyte apoptosis may explain the reduction of total BM

megakaryocytes.

Together, these preliminary results suggest that CIH followed by a normoxia period changes the BM vascular compartment by increasing $CD105^+$ vessel coverage, decreasing VE-$Cadherin^+$ and SMA^+ vessel density, decreasing total megakaryocyte numbers and promoting megakaryocyte terminal differentiation.

Chronic intermittent hypoxia modulates bone marrow "angiocrine" gene expression

Next, we searched for modulation of "angiocrine" genes, since these were considered to be the effectors for the instructive role of the BM vascular niche [44,45,55].

Our preliminary data strongly suggest an upregulation of BM IL-6 expression in the BM of CIH-treated rats. There may also be an upregulation of Dll1, CSF2, CSF3, Smad3 and THPO, and a downregulation of IGF1, IGFbp3, IGFbp5, Angpt1, SCF and N-Cadherin expression in the CIH-treated rats (Figure 4.5).

Interleukin 6 is reported to be upregulated by hypoxia in endothelial cells *in vitro* and in lung vessels of mice subjected to chronic hypoxia [46,102]. Angiopoietin 1 is reported to be downregulated by hypoxia in glioblastoma cells *in vitro* [1,3,6-9,11,13,14,16,42,103]. Both Dll1 and N-Cadherin are upregulated upon hypoxia in ovarian carcinoma cells [2,104]. *In vitro* studies have revealed that hypoxic responses in both endothelial cells and BM stromal cells are mediated by Smad3 activation [47,105,106], even though we could not find any report of the modulation of Smad3 mRNA expression under hypoxic conditions.

In spite of the decrease of SCF, which promotes the proliferation of progenitor erythrocytes [48,107], IL-6 was previously shown to promote erythroid differentiation *in vitro* [49,108]. However, the major driver of erythropoiesis is probably erythropoietin [30,109].

Interestingly, and even though there may be a decrease in IGF1, IGFbp3, IGFbp5 and SCF, which are known to promote proliferation and differentiation of myeloid lineage cells [50,110-112], the possible increase of IL-6, CSF2 and CSF3 may contribute to an increase in monocyte counts [51,113-115].

Thrombopoietin increases platelet counts and megakaryocyte numbers *in vivo* [30,116-119]. Interleukin 6 and CSF2 enhance megakaryocyte adhesion to endothelial cells [52-54,120], which is indispensable for the latest steps of megakaryocyte differentiation and platelet release [55,121]. It is particularly interesting that IL-6 was shown to promote thrombocytosis with an increase of megakaryocyte size without altering megakaryocyte number [59,122], the same observations as those reported in both acute and short-term chronic hypoxia (4 days) [1,3,6,7,9,14,16,24,42,61].

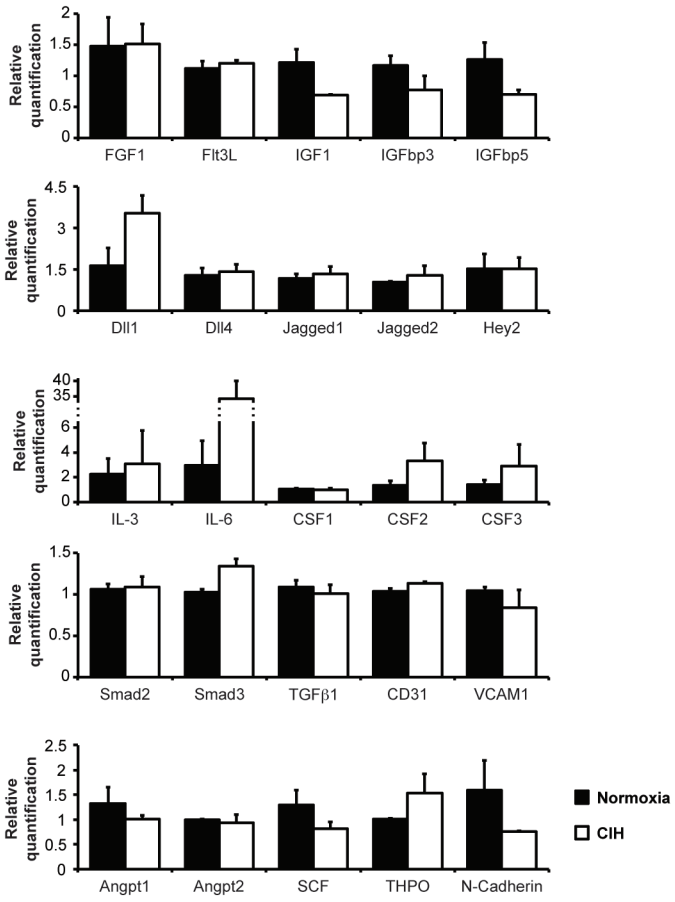


Figure 4.5. Chronic intermittent hypoxia modulates bone marrow angiocrine gene expression.
Angiocrine gene modulation was assessed by relative quantification of mRNA from total BM. The data suggest an increase in IL-6, Dll1, CSF2, CSF3, Smad3 and THPO and a decrease in IGF1, IGFbp3, IGFbp5, Angpt1, SCF and N-Cadherin expression.
Data are means ± s.e.m. n=2.

Together, these preliminary data suggest that CIH followed by a normoxia period modulates BM "angiocrine" gene expression.

DISCUSSION

The preliminary data reported in this chapter (obtained from 2 rats per condition, thus not a statistical sample) suggest CIH followed by a normoxia period increases erythropoiesis and promotes monocytosis and thrombocytosis and modulates the BM vascular compartment. These results further suggest CIH may promote megakaryocyte terminal differentiation. The possible hematopoietic modulation may be partially driven by "angiocrine" gene variations in the BM of the rats exposed to CIH.

The strongest "angiocrine" gene candidate for the hematopoietic changes herein suggested is IL-6, which according to our preliminary results may be up to 10 times more expressed in the CIH animals than normoxic controls (Figure 4.5). Interleukin 6 was previously reported to up-regulate the expression of functional CSF1 receptor in monocytes, thus allowing autocrine CSF1 signaling, which in turn promotes monocyte proliferation and survival [4,57,123-125]. Furthermore, IL-6 acts synergistically with other growth factors to induce the proliferation of HSCs, granulocyte/macrophage progenitors and colony forming unit-monocytes [30,50,113,114,126,127]. Therefore, IL-6 may regulate monocyte numbers by expanding stem, progenitor and mature cells.

As previously suggested, the possible thrombocytosis evidenced by these preliminary data may be explained by the increase of IL-6 and THPO. It may also be explained by a possible increased blood shear rate in this model of obstructive sleep apnea. Chronic hypoxia was reported to modulate platelets in a oscillatory fashion, first increasing (days 2-4) then decreasing (days 6-14) its numbers [8,9,11,60]. Interestingly, a transient increase of blood flow rate was previously observed in both acute hypoxia and the first 4 days of chronic hypoxic stimulus [12,18,29,128-130]. The increase of blood flow rate is related to an increased shear rate [8,9,24,61,131]. It was previously shown that exposure of megakaryocytes *in vitro* to high shear rates accelerates platelet production [8,9,11,24,132]. This would

sustain McDonald's proposal that the initial rapid increase of platelets in chronic hypoxia is due to the release of platelets to the periphery [9,24,57]. This release of platelets may then deplete mature BM megakaryocytes [8,13,24,133], which together with the fact that at least *in vitro* hypoxia delays megakaryopoiesis [20,50,59,62-64] can explain the thrombocytopenia observed in later stages of chronic hypoxia (days 6-14) [6-9,11]. However, our preliminary results suggest that 32 days of CIH followed by a normoxic period may increase platelet numbers. Interestingly, obstructive sleep apnea results in an increased blood flow velocity [46,134]. If the used model of obstructive sleep apnea also mimics the disease in regard to blood flow velocity, and the normoxia period after CIH increase pO_2 in the BM [46,65-68], thereby regulating megakaryocytosis kinetics [20], the possible thrombocytosis evidenced by the preliminary data herein presented may be explained by an increased shear rate.

Avecilla and colleagues previously observed a correlation between VE-Cadherin$^+$ vessels and megakaryocyte numbers in the BM [121]. Interestingly, the same kinetics may to apply to our system, with a reduction in both megakaryocyte numbers and VE-Cadherin$^+$ vessel numbers. Furthermore, the number of apoptotic megakaryocytes seems to be increased in CIH-subjected rats, which, as previously mentioned, may be related to a normalization of megakaryopoiesis kinetics in the post-hypoxic period.

We found evidence of a possible modulation of the BM vascular compartment by CIH. To our knowledge, the proposal that hypoxia may modulate the BM vascular compartment has only been assessed in 1974, with observations that led to the suggestion of a BM vascular niche remodeling [135]. In this electron microscopy study, the only characteristic which varied between the experimental groups was the increase of occasional gaps in the sinus wall which were plugged by platelets and fibrin in hypoxia-subjected animals [135]. Interestingly, this paper referred to short-term chronic hypoxia (up to 5 days), and supports McDonald's

theory of the increased platelet release in the first days of chronic hypoxia [9]. These results and our preliminary data suggest hypoxia modulates the BM vasculature, which may promote the hematopoietic changes induced by hypoxia.

The preliminary results herein shown do not yet include HSPCs measurements. However, these will be very interesting to assess, as relevant clinical data for both understanding obstructive sleep apnea and therapeutically improving BM transplants can arise. Previous reports show that in mice subjected to chronic hypoxia BM HSPC numbers are unaffected, however, they increase during the posthypoxic period [3,10,42]. Consequently, hypoxia has raised significant interest as a treatment for preparation of myeloablative irradiation. Bone marrow cells cultured in hypoxia are more resistant to ionizing radiation and promote a faster hematopoietic recovery after transplantation, despite a reduction in HPC numbers in hypoxic cultures [15]. In fact, *in vivo* treatment with hypoxia before irradiation accelerates the recovery of HSPCs and myeloid, but not lymphoid or erythrocytes, cells [5]. Furthermore, both genetic and pharmacologic modulation of HIF-α in hematopoietic cells reveal a precise modulation of HIF-1α improves hematopoietic recovery following bone marrow transplant [26,28,29]. Thus, the exploration of different hypoxia posology as therapeutic assessments for improved hematopoietic recovery in bone marrow transplants may yield interesting pre-clinical results.

Our preliminary data suggest that CIH followed by a normoxic period may increase erythropoiesis, blood monocyte counts and thrombopoiesis. Our characterization of the BM vascular compartment suggests CIH increases the $CD105^+$ vessel density and reduces VE-$Cadherin^+$ and SMA^+ vessel number without affecting vWF^+ vessels, and reduces total BM megakaryocyte numbers and increases megakaryocyte terminal differentiation. Furthermore, CIH modulates several "angiocrine" genes expression in the BM. The completion of this study may better

elucidate the hematopoietic changes after CIH, which may have clinical value not only for obstructive sleep apnea, but also for bone marrow transplants.

REFERENCES

1. Reissmann, K. R. Studies on the mechanism of erythropoietic stimulation in parabiotic rats during hypoxia. *Blood* 5, 372–380 (1950).

2. Fisher, J. W. Landmark advances in the development of erythropoietin. *Exp. Biol. Med. (Maywood)* 235, 1398–1411 (2010).

3. Bruce, W. R. & McCulloch, E. A. The Effect of Erythropoietic Stimulation on the Hemopoietic Colony-forming Cell of Mice. *Blood* 23, 216–232 (1964).

4. Boyum, A., Carsten, A. L., Laerum, O. D. & Cronkite, E. P. Kinetics of cell proliferation of murine bone marrow cells cultured in diffusion chambers: effect of hypoxia, bleeding, erythropoietin injections, polycythemia, and irradiation of the host. *Blood* 40, 174–188 (1972).

5. Beran, M. Hemopoietic recovery in posthypoxic mice: repopulation of CFUs and morphologically identifiable cells in the bone marrow and spleen. *Radiat. Res.* 53, 468–479 (1973).

6. Murphy, M. J. & Lord, B. I. Hematopoietic stem cell regulation. I. Acute effects of hypoxic-hypoxia on CFU kinetics. *Blood* 42, 81–87 (1973).

7. Lord, B. I. & Murphy, M. J. Hematopoietic stem cell regulation. II. Chronic effects of hypoxic-hypoxia on CFU kinetics. *Blood* 42, 89–98 (1973).

8. Jackson, C. W. & Edwards, C. C. Biphasic thrombopoietic response to severe hypobaric hypoxia. *Br. J. Haematol.* 35, 233–244 (1977).

9. McDonald, T. P., Cottrell, M. & Clift, R. Effects of short-term hypoxia on platelet counts of mice. *Blood* 51, 165–175 (1978).

10. Loeffler, M., Herkenrath, P., Wichmann, H. E., Lord, B. I. & Murphy, M. J. The kinetics of hematopoietic stem cells during and after hypoxia. A model analysis. *Blut* 49, 427–439 (1984).

11. Petursson, S. R. & Chervenick, P. A. Effects of hypoxia on megakaryocytopoiesis and granulopoiesis. *Eur. J. Haematol.* 39, 267–273 (1987).

12. Koller, M. R., Bender, J. G., Miller, W. M. & Papoutsakis, E. T. Reduced oxygen tension increases hematopoiesis in long-term culture of human stem and progenitor cells from cord blood and bone marrow. *Exp. Hematol.* 20, 264–270 (1992).

13. McDonald, T. P. *et al.* Comparison of platelet production in two strains of mice with different modal megakaryocyte DNA ploidies after exposure to hypoxia. *Exp. Hematol.* 20, 51–56 (1992).

14. McDonald, T. P., Cottrell, M. B., Clift, R. E. & Jackson, C. W. Effects of hypoxia on megakaryocyte size and number of C3H and BALB/c mice. *Proc. Soc. Exp. Biol. Med.* 199, 287–290 (1992).

15. Cipolleschi, M. G., Sbarba, Dello, P. & Olivotto, M. The role of hypoxia in the maintenance of hematopoietic stem cells. *Blood* 82, 2031–2037 (1993).

16. McGuire, M. & Bradford, A. Chronic intermittent hypoxia increases haematocrit and causes right ventricular hypertrophy in the rat. *Respir Physiol* 117, 53–58 (1999).

17. Ivanovi , Z. *et al.* Incubation of murine bone marrow cells in hypoxia ensures the maintenance of marrow-repopulating ability together with the expansion of committed progenitors. *Br. J. Haematol.* 108, 424–429 (2000).

18. Hevehan, D. L., Papoutsakis, E. T. & Miller, W. M. Physiologically significant effects of pH and oxygen tension on granulopoiesis. *Exp. Hematol.* 28, 267–275 (2000).

19. Hevehan, D. L., Wenning, L. A., Miller, W. M. & Papoutsakis, E. T. Dynamic model of ex vivo granulocytic kinetics to examine the effects of oxygen tension, pH, and interleukin-3. *Exp. Hematol.* 28, 1016–1028 (2000).

20. Mostafa, S. S., Miller, W. M. & Papoutsakis, E. T. Oxygen tension influences the differentiation, maturation and apoptosis of human megakaryocytes. *Br. J. Haematol.* 111, 879–889 (2000).

21. Chow, D. C., Wenning, L. A., Miller, W. M. & Papoutsakis, E. T. Modeling pO(2) distributions in the bone marrow hematopoietic compartment. I. Krogh's model. *Biophys. J.* 81, 675–684 (2001).

22. Chow, D. C., Wenning, L. A., Miller, W. M. & Papoutsakis, E. T. Modeling pO(2) distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models. *Biophys. J.* 81, 685–696 (2001).

23. Huang, Y. *et al.* Cobalt chloride and low oxygen tension trigger differentiation of acute myeloid leukemic cells: possible mediation of hypoxia-inducible factor-1α. *Leukemia* 17, 2065–2073 (2003).

24. Lebedeva, E. V., Yushkov, B. G. & Chereshnev, V. A. Megakaryocytopoiesis under hypoxic conditions. *Bull. Exp. Biol. Med.* 136, 554–556 (2003).

25. Jing, D. *et al.* Oxygen tension plays a critical role in the hematopoietic microenvironment in vitro. *Haematologica* 97, 331–339 (2012).

26. Takubo, K. *et al.* Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. *Cell Stem Cell* 7, 391–402 (2010).

27. Simsek, T. *et al.* The Distinct Metabolic Profile of Hematopoietic Stem Cells Reflects Their Location in a Hypoxic Niche. *Stem Cell* 7, 380–390 (2010).

28. Rehn, M. *et al.* Hypoxic induction of vascular endothelial growth factor regulates murine hematopoietic stem cell function in the low-oxygenic niche. *Blood* 118, 1534–1543 (2011).

29. Forristal, C. E. *et al.* Pharmacologic stabilization of HIF-1α increases hematopoietic stem cell quiescence in vivo and accelerates blood recovery after severe irradiation. *Blood* 121, 759–769 (2013).

30. Eisensehr, I. & Noachtar, S. Haematological aspects of obstructive sleep apnoea. *Sleep Med Rev* 5, 207–221 (2001).

31. Bradley, T. D. & Floras, J. S. Obstructive sleep apnoea and its cardiovascular consequences. *Lancet* 373, 82–93 (2009).

32. Allalunis, M. J., Chapman, J. D. & Turner, A. R. Identification of a hypoxic population of bone marrow cells. *Int. J. Radiat. Oncol. Biol. Phys.* 9, 227–232 (1983).

33. Parmar, K., Mauch, P., Vergilio, J.-A., Sackstein, R. & Down, J. D. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5431–5436 (2007).

34. Levesque, J.-P. *et al.* Hematopoietic Progenitor Cell Mobilization Results in Hypoxia with Increased Hypoxia-Inducible Transcription Factor-1α and Vascular Endothelial Growth Factor A in Bone Marrow. *Stem Cells* 25, 1954–1965 (2007).

35. Kubota, Y., Takubo, K. & Suda, T. Bone marrow long label-retaining cells reside in the sinusoidal hypoxic niche. *Biochemical and Biophysical Research Communications* 366, 335–339 (2008).

36. Weigert, A. *et al.* HIF-1α is a negative regulator of plasmacytoid DC development in vitro and in vivo. *Blood* 120, 3001–3006 (2012).

37. Cooper, B. The origins of bone marrow as the seedbed of our blood: from antiquity to the time of Osler. *Proc (Bayl Univ Med Cent)* 24, 115–118 (2011).

38. Winkler, I. G. *et al.* Positioning of bone marrow hematopoietic and stromal cells relative to blood flow in vivo: serially reconstituting hematopoietic stem cells reside in distinct nonperfused niches. *Blood* 116, 375–385 (2010).

39. Kalra, V. K., Shen, Y., Sultana, C. & Rattan, V. Hypoxia induces PECAM-1 phosphorylation and transendothelial migration of monocytes. *Am. J. Physiol.* 271, H2025–34 (1996).

40. Tøndevold, E., Eriksen, J. & Jansen, E. Observations on long bone medullary pressures in relation to arterial PO2, PCO2 and pH in the anaesthetized dog. *Acta Orthop Scand* 50, 645–651 (1979).

41. Harrison, J. S., Rameshwar, P., Chang, V. & Bandari, P. Oxygen saturation in the bone marrow of healthy volunteers. *Blood* 99, 394 (2002).

42. Kubanek, B. *et al.* Regulation of erythropoiesis: XXIII. Dissociation between stem cell and erythroid response to hypoxia. *Blood* 32, 586–596 (1968).

43. Semenza, G. L. Hypoxia-Inducible Factors in Physiology and Medicine. *Cell* 148, 399–408 (2012).

44. Cramer, T. *et al.* HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell* 112, 645–657 (2003).

45. Pedersen, M. *et al.* Stem cell factor induces HIF-1α at normoxia in hematopoietic cells. *Biochemical and Biophysical Research Communications* 377, 98–103 (2008).

46. Eriksen, J., Tøndevold, E., Jansen, E. & Petersen, J. E. Relationships between oxygen and carbon dioxide tensions and acid-base balance in arterial blood and in medullary blood from long bones in dogs. *Acta Orthop Scand* 50, 519–525 (1979).

47. Kopp, H.-G., Avecilla, S. T., Hooper, A. T. & Rafii, S. The bone marrow vascular niche: home of HSC differentiation and mobilization. *Physiology (Bethesda)* 20, 349–356 (2005).

48. Pugh, C. W. & Ratcliffe, P. J. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nature Medicine* 9, 677–684 (2003).

49. Fletcher, E. C., Lesske, J., Qian, W., Miller, C. C. & Unger, T. Repetitive, episodic hypoxia causes diurnal elevation of blood pressure in rats. *Hypertension* 19, 555–561 (1992).

50. Hoffstein, V., Herridge, M., Mateika, S., Redline, S. & Strohl, K. P. Hematocrit levels in sleep apnea. *Chest* 106, 787–791 (1994).

51. Krieger, J., Sforza, E., Barthelmebs, M., Imbs, J. L. & Kurtz, D. Overnight decrease in hematocrit after nasal CPAP treatment in patients with OSA. *Chest* 97, 729–730 (1990).

52. Varghese, A. J., Gulyas, S. & Mohindra, J. K. Hypoxia-dependent reduction of 1-(2-nitro-1-imidazolyl)-3-methoxy-2-propanol by Chinese hamster ovary cells and KHT tumor cells in vitro and in vivo. *Cancer Research* 36, 3761–3765 (1976).

53. Sheldon, P. W., Batten, E. L. & Adams, G. E. Potentiation of melphalan activity against a murine tumour by nitroimidazole compounds. *Br. J. Cancer* 46, 525–531 (1982).

54. Raleigh, J. A. & Koch, C. J. Importance of thiols in the reductive binding of 2-nitroimidazoles to macromolecules. *Biochem. Pharmacol.* 40, 2457–2464 (1990).

55. Kobayashi, H. *et al.* Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nature Cell Biology* 12, 1046–1056 (2010).

56. LaManna, J. C., Vendel, L. M. & Farrell, R. M. Brain adaptation to chronic hypobaric hypoxia in rats. *J Appl Physiol* 72, 2238–2243 (1992).

57. Dunleavy, M., Dooley, M., Cox, D. & Bradford, A. Chronic intermittent asphyxia increases platelet reactivity in rats. *Exp. Physiol.* 90, 411–416 (2005).

58. Liu, W. *et al.* Induction of tumor arrest and differentiation with prolonged survival by intermittent hypoxia in a mouse model of acute myeloid leukemia. *Blood* 107, 698–707 (2006).

59. Varol, E. *et al.* Mean platelet volume is increased in patients with severe obstructive sleep apnea. *Scand. J. Clin. Lab. Invest.* 70, 497–502 (2010).

60. Coates, G., Nahmias, C. & Thind, A. Platelets and leukocytes in the lungs after acute hypobaric hypoxia. *J Appl Physiol* 55, 1536–1541 (1983).

61. Hodkinson, P. D., Hunt, B. J., Parmar, K. & Ernsting, J. Is mild normobaric hypoxia a risk factor for venous thromboembolism? *J. Thromb. Haemost.* 1, 2131–2133 (2003).

62. Bokinsky, G., Miller, M., Ault, K., Husband, P. & Mitchell, J. Spontaneous platelet activation and aggregation during obstructive sleep apnea and its response to therapy with nasal continuous positive airway pressure. A preliminary investigation. *Chest* 108, 625–630 (1995).

63. Vagdatli, E. *et al.* Platelet distribution width: a simple, practical and specific marker of activation of coagulation. *Hippokratia* 14, 28–32 (2010).

64. Nena, E. *et al.* Mean Platelet Volume and Platelet Distribution Width in non-diabetic subjects with obstructive sleep apnoea syndrome: new indices of severity? *Platelets* 23, 447–454 (2012).

65. Candal, F. J. *et al.* BMEC-1: a human bone marrow microvascular endothelial cell line with primary cell characteristics. *Microvasc. Res.* 52, 221–234 (1996).

66. Zhang, Y. *et al.* Tie2Cre-mediated inactivation of plexinD1 results in congenital heart, vascular and skeletal defects. *Developmental Biology* 325, 82–93 (2009).

67. Hooper, A. T. *et al.* Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. *Cell Stem Cell* 4, 263–274 (2009).

68. Ding, L., Saunders, T. L., Enikolopov, G. & Morrison, S. J. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 481, 457–462 (2012).

69. Skalli, O. *et al.* Alpha-smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericytes. *J. Histochem. Cytochem.* 37, 315–321 (1989).

70. Galmiche, M. C., Koteliansky, V. E., Brière, J., Hervé, P. & Charbord, P. Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway. *Blood* 82, 66–76 (1993).

71. Bianco, P., Riminucci, M., Gronthos, S. & Robey, P. G. Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells* 19, 180–192 (2001).

72. Opitz, E. Increased vascularization of the tissue due to acclimatization to high altitude and its significance for the oxygen transport. *Exp Med Surg* 9, 389–403 (1951).

73. Miller, A. T. & Hale, D. M. Increased vascularity of brain, heart, and skeletal muscle of polycythemic rats. *Am. J. Physiol.* 219, 702–704 (1970).

74. Knighton, D. R. *et al.* Oxygen tension regulates the expression of angiogenesis factor by macrophages. *Science* 221, 1283–1285 (1983).

75. Adair, T. H., Gay, W. J. & Montani, J. P. Growth regulation of the vascular system: evidence for a metabolic hypothesis. *Am. J. Physiol.* 259, R393–404 (1990).

76. Shweiki, D., Itin, A., Soffer, D. & Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359, 843–845 (1992).

77. Goldberg, M. A. & Schneider, T. J. Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *J. Biol. Chem.* 269, 4355–4359 (1994).

78. Harik, S. I., Hritz, M. A. & LaManna, J. C. Hypoxia-induced brain angiogenesis in the adult rat. *J. Physiol. (Lond.)* 485 (Pt 2), 525–530 (1995).

79. Harik, N. *et al.* Time-course and reversibility of the hypoxia-induced alterations in cerebral vascularity and cerebral capillary glucose transporter density. *Brain Res.* 737, 335–338 (1996).

80. Patt, S., Sampaolo, S., Théallier-Jankó, A., Tschairkin, I. & Cervós-Navarro, J. Cerebral angiogenesis triggered by severe chronic hypoxia displays regional differences. *J. Cereb. Blood Flow Metab.* 17, 801–806 (1997).

81. Boero, J. A., Ascher, J., Arregui, A., Rovainen, C. & Woolsey, T. A. Increased brain capillaries in chronic hypoxia. *J Appl Physiol* 86, 1211–1219 (1999).

82. Howell, K., Preston, R. J. & McLoughlin, P. Chronic hypoxia causes angiogenesis in addition to remodelling in the adult rat pulmonary circulation. *J. Physiol. (Lond.)* 547, 133–145 (2003).

83. Saldívar, E., Cabrales, P., Tsai, A. G. & Intaglietta, M. Microcirculatory changes during chronic adaptation to hypoxia. *Am. J. Physiol. Heart Circ. Physiol.* 285, H2064–71 (2003).

84. Rofstad, E. K., Gaustad, J.-V., Egeland, T. A. M., Mathiesen, B. & Galappathi, K. Tumors exposed to acute cyclic hypoxic stress show enhanced angiogenesis, perfusion and metastatic dissemination. *Int. J. Cancer* 127, 1535–1546 (2010).

85. Gaustad, J.-V., Simonsen, T. G., Roa, A. M. A. & Rofstad, E. K. Tumors exposed to acute cyclic hypoxia show increased vessel density and delayed blood supply. *Microvasc. Res.* (2012). doi:10.1016/j.mvr.2012.11.002

86. Sánchez-Elsner, T., Botella, L. M., Velasco, B., Langa, C. & Bernabéu, C. Endoglin expression is regulated by transcriptional cooperation between the hypoxia and transforming growth factor-beta pathways. *J. Biol. Chem.* 277, 43799–43808 (2002).

87. Li, C. *et al.* CD105 prevents apoptosis in hypoxic endothelial cells. *Journal of Cell Science* 116, 2677–2685 (2003).

88. Lebrin, F. *et al.* Endoglin promotes endothelial cell proliferation and TGF-β/ALK1 signal transduction. *EMBO J.* 23, 4018–4028 (2004).

89. Remédio, L. *et al.* Context- and cell-dependent effects of delta-like 4 targeting in the bone marrow microenvironment. *PLoS ONE* 7, e52450 (2012).

90. Li, T.-S. *et al.* Improved angiogenic potency by implantation of ex vivo hypoxia prestimulated bone marrow cells in rats. *Am. J. Physiol. Heart Circ. Physiol.* 283, H468–73 (2002).

91. Wojciak-Stothard, B., Tsang, L. Y. F. & Haworth, S. G. Rac and Rho play opposing roles in the regulation of hypoxia/reoxygenation-induced permeability changes in pulmonary artery endothelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* 288, L749–60 (2005).

92. Quinlan, T. R. *et al.* eNOS-deficient mice show reduced pulmonary vascular proliferation and remodeling to chronic hypoxia. *Am. J. Physiol. Lung Cell Mol. Physiol.* 279, L641–50 (2000).

93. Dore-Duffy, P. & LaManna, J. C. Physiologic angiodynamics in the brain. *Antioxid. Redox Signal.* 9, 1363–1371 (2007).

94. Kuwabara, K. *et al.* Hypoxia-mediated induction of acidic/basic fibroblast growth factor and platelet-derived growth factor in mononuclear phagocytes stimulates growth of hypoxic endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4606–4610 (1995).

95. Gleadle, J. M., Ebert, B. L., Firth, J. D. & Ratcliffe, P. J. Regulation of angiogenic growth factor expression by hypoxia, transition metals, and chelating agents. *Am. J. Physiol.* 268, C1362–8 (1995).

96. Norman, J. T., Clark, I. M. & Garcia, P. L. Hypoxia promotes fibrogenesis in human renal fibroblasts. *Kidney Int.* 58, 2351–2366 (2000).

97. Short, M., Nemenoff, R. A., Zawada, W. M., Stenmark, K. R. & Das, M. Hypoxia induces differentiation of pulmonary artery adventitial fibroblasts into myofibroblasts. *Am. J. Physiol., Cell Physiol.* 286, C416–25 (2004).

98. Chen, Y.-F. *et al.* Dominant negative mutation of the TGF-beta receptor blocks hypoxia-induced pulmonary vascular remodeling. *J Appl Physiol* 100, 564–571 (2006).

99. Modarressi, A. *et al.* Hypoxia impairs skin myofibroblast differentiation and function. *J. Invest. Dermatol.* 130, 2818–2827 (2010).

100. Piovella, F. *et al.* Immunofluorescent Detection of Factor VIII-Related Antigen in Human Platelets and Megakaryocytes. *Haemostasis* 3, 288–295 (1974).

101. Gordge, M. P. Megakaryocyte apoptosis: sorting out the signals. *Br. J. Pharmacol.* 145, 271–273 (2005).

102. Yan, S. F. *et al.* Induction of interleukin 6 (IL-6) by hypoxia in vascular cells. Central role of the binding site for nuclear factor-IL-6. *J. Biol. Chem.* 270, 11463–11471 (1995).

103. Enholm, B. *et al.* Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia. *Oncogene* 14, 2475–2483 (1997).

104. Sahlgren, C., Gustafsson, M. V., Jin, S., Poellinger, L. & Lendahl, U. Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proc Natl Acad Sci U S A* 105, 6392–6397 (2008).

105. Zhang, H. *et al.* Cellular response to hypoxia involves signaling via Smad proteins. *Blood* 101, 2253–2260 (2003).

106. Zhou, S., Lechpammer, S., Greenberger, J. S. & Glowacki, J. Hypoxia inhibition of adipocytogenesis in human bone marrow stromal cells requires transforming growth factor-beta/Smad3 signaling. *J. Biol. Chem.* 280, 22688–22696 (2005).

107. Broudy, V. C. Stem cell factor and hematopoiesis. *Blood* 90, 1345–1364 (1997).

108. Suzuki, M. *et al.* Delta-4 expression on a stromal cell line is augmented by interleukin-6 via STAT3 activation. *Exp. Hematol.* 34, 1142–1149 (2006).

109. Jelkmann, W. Control of erythropoietin gene expression and its use in medicine. *Meth. Enzymol.* 435, 179–197 (2007).

110. Tian, Z. G. *et al.* Recombinant human growth hormone promotes hematopoietic reconstitution after syngeneic bone marrow transplantation in mice. *Stem Cells* 16, 193–199 (1998).

111. Heemskerk, V. H., Daemen, M. A. & Buurman, W. A. Insulin-like growth factor-1 (IGF-1) and growth hormone (GH) in immunity and inflammation. *Cytokine Growth Factor Reviews* 10, 5–14 (1999).

112. Takagi, S. *et al.* Membrane-bound human SCF/KL promotes in vivo human hematopoietic engraftment and myeloid differentiation. *Blood* (2012). doi:10.1182/blood-2011-05-353201

113. Ikebuchi, K. *et al.* Interleukin 6 enhancement of interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. *Proc. Natl. Acad. Sci. U.S.A.* 84, 9035–9039 (1987).

114. Koike, K. *et al.* Synergism of BSF-2/interleukin 6 and interleukin 3 on development of multipotential hemopoietic progenitors in serum-free culture. *J. Exp. Med.* 168, 879–890 (1988).

115. Metcalf, D. The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors. *Blood* 67, 257–267 (1986).

116. Kaushansky, K. *et al.* Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. *Nature* 369, 568–571 (1994).

117. de Sauvage, F. J. *et al.* Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. *Nature* 369, 533–538 (1994).

118. Bartley, T. D. *et al.* Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. *Cell* 77, 1117–1124 (1994).

119. Sitnicka, E. *et al.* The effect of thrombopoietin on the proliferation and differentiation of murine hematopoietic stem cells. *Blood* 87, 4998–5005 (1996).

120. Avraham, H., Cowley, S., Chi, S. Y., Jiang, S. & Groopman, J. E. Characterization of adhesive interactions between human endothelial cells and megakaryocytes. *J. Clin. Invest.* 91, 2378–2384 (1993).

121. Avecilla, S. T. *et al.* Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nature Medicine* 10, 64–71 (2004).

122. Ishibashi, T. *et al.* Interleukin-6 is a potent thrombopoietic factor in vivo in mice. *Blood* 74, 1241–1244 (1989).

123. Elliott, M. J. *et al.* Recombinant human interleukin-3 and granulocyte-macrophage colony-stimulating factor show common biological effects and binding characteristics on human monocytes. *Blood* 74, 2349–2359 (1989).

124. Cheung, D. L. & Hamilton, J. A. Regulation of human monocyte DNA synthesis by colony-stimulating factors, cytokines, and cyclic adenosine monophosphate. *Blood* 79, 1972–1981 (1992).

125. Chomarat, P., Banchereau, J., Davoust, J. & Palucka, A. K. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat. Immunol.* 1, 510–514 (2000).

126. Leary, A. G. *et al.* Synergism between interleukin-6 and interleukin-3 in supporting proliferation of human hematopoietic stem cells: comparison with interleukin-1 alpha. *Blood* 71, 1759–1763 (1988).

127. Brugger, W. *et al.* Ex vivo expansion of enriched peripheral blood CD34+ progenitor cells by stem cell factor, interleukin-1 beta (IL-1 beta), IL-6, IL-3, interferon-gamma, and erythropoietin. *Blood* 81, 2579–2584 (1993).

128. Bereczki, D. *et al.* Hypoxia increases velocity of blood flow through parenchymal microvascular systems in rat brain. *J. Cereb. Blood Flow Metab.* 13, 475–486 (1993).

129. Wood, J. G., Johnson, J. S., Mattioli, L. F. & Gonzalez, N. C. Systemic hypoxia promotes leukocyte-endothelial adherence via reactive oxidant generation. *J Appl Physiol* 87, 1734–1740 (1999).

130. Xu, K., Puchowicz, M. A. & LaManna, J. C. Renormalization of regional brain blood flow during prolonged mild hypoxic exposure in rats. *Brain Res.* 1027, 188–191 (2004).

131. Papaioannou, T. G. & Stefanadis, C. Vascular wall shear stress: basic principles and methods. *Hellenic J Cardiol* 46, 9–15 (2005).

132. Dunois-Lardé, C. *et al.* Exposure of human megakaryocytes to high shear rates accelerates platelet production. *Blood* 114, 1875–1883 (2009).

133. Kobayashi, Y. & Kondo, M. Human megakaryocyte ploidy. *Histol. Histopathol.* 14, 1223–1229 (1999).

134. Erdem, C. Z. *et al.* Doppler measurement of blood flow velocities in extraocular orbital vessels in patients with obstructive sleep apnea syndrome. *J Clin Ultrasound* 31, 250–257 (2003).

135. Ben-Ishay, Z. Sinus repair in the bone marrow of hypoxic mice. Possible relationship to cellular migration. *Scand J Haematol* 12, 37–45 (1974).

5
DISCUSSION

CONTENTS

THE CONSEQUENCES OF SYSTEMIC DLL4 BLOCKADE IN THE BM MICROENVIRONMENT AND HEMATOPOIESIS 131

THE ROLE OF CHRONIC INTERMITTENT HYPOXIA, A MODEL FOR OBSTRUCTIVE SLEEP APNEA, IN THE BM MICROENVIRONMENT AND HEMATOPOIESIS 139

CONCLUDING REMARKS 143

REFERENCES 145

The association between blood vessels and hematopoietic cells in the bone marrow (BM) was first reported in the late 19th century, less than 30 years after the identification of the BM as the primary site for hematopoiesis and more than 80 years before the first demonstration that BM stromal cells support hematopoiesis [1-6]. Over 100 years later, the work included in this dissertation began, in a time when BM endothelial cells (ECs) were mainly studied as a uniform population of cells lining the blood vessels. During this period, we and others have revealed the importance of the vascular heterogeneity in the BM, and scrutinized its relevance in terms of hematopoietic modulation [7-13]. The research on the cross-talk between different BM vessels and hematopoietic cells contribute to a better understanding of hematopoiesis, and may result in interesting clinical applications.

In this Thesis, we used two strategies to modulate the BM vascular niche and to characterize its consequences for hematopoiesis. First, we employed a "therapeutic" (systemic) approach, consisting of delta-like (Dll)4 blockade using specific neutralizing antibodies. We started with the hypothesis that Dll4 blockade would primarily affect ECs, thereby modulating vessel formation and function. This strategy was at first systemic, but we further explored the specificity of the effects of Dll4 blockade on ECs by targeting the vascular endothelial cadherin (VE-Cadherin)$^{+}$ ECs in VECad^{CreERT2}Dll4$^{lox/lox}$. The second strategy consisted in modulating the macroenvironment using a model for obstructive sleep apnea, chronic intermittent hypoxia (CIH). Hypoxia had been shown to modulate blood vessels and hematopoiesis, however, clinical data from obstructive sleep apnea patients are contradictory and the cross talk between BM ECs and hematopoietic cells in hypoxic conditions *in vivo* were unknown.

THE CONSEQUENCES OF SYSTEMIC DLL4 BLOCKADE IN THE BM MICROENVIRONMENT AND HEMATOPOIESIS

At the beginning of this project, Dll4 was thought to be primarily expressed, in the adult, in small arteries and microvessels, by tip cells (mostly in angiogenic blood vessels) [14-18], and was later shown, in the BM microenvironment, to be largely restricted to blood vessels [5,19]. Delta-like 4 blockade in solid tumors was shown to induce EC proliferation and enhance sprouting and branching, resulting in increased vessel coverage rendering poor perfusion and increased hypoxia [16,17,20,21]. However, the role of Dll4/Notch signaling in the BM vasculature was completely unknown.

The most important site of hematopoietic cell differentiation and mobilization, to the peripheral blood or into the BM, is the vascular sinusoidal bed [22-25], which is characterized by its poor perfusion (due to low blood flow rate) [16,22,26]. Thus, we reasoned that Dll4 blockade could result in an increased number of functional BM vessels in a setting of BM remodeling (when there should be more "tip cells", thus, more Dll4). The BM vessels regress after myeloablation, and their assembly and remodeling are crucial for hematopoietic recovery [27-30]. Our original hypothesis was that administration of anti-Dll4 post-myeloablation would target specifically ECs and increase the vascular coverage, thus accelerating hematopoietic recovery.

Surprisingly, unlike in tumor models, Dll4 blockade did not increase the overall BM vessel content, but rather shifted EC identity (Figures 2.1, 2.6), which did not result in changes of vessel type (Figures 2.2, 2.3). These domains of BM EC "markers" expression had been largely unnoticed, and may have a large impact in understanding the cross-talk between endothelial and hematopoietic cells, and even for the definition of specific BM hematopoietic "niches". As discussed in Chapter

2, the EC markers that varied with anti-Dll4 treatment, VE-Cadherin and CD31, were previously shown to have specific roles in BM hematopoiesis: VE-Cadherin negatively mediates transendothelial migration of HSPCs and CD31 is required for HSPCs and neutrophil transendothelial migration [31-36], both VE-Cadherin and CD31 are required for thrombopoiesis [37-40], and CD31 is required for leukocyte adhesion to ECs [40]. Thus, the vascular heterogeneity that we report is likely to create different microenvironments with distinct abilities to support specific stages of hematopoiesis, or different hematopoietic lineages. In fact, during the preparation of this dissertation two papers have further demonstrated BM vascular heterogeneity independently of vessel type. They show that E-selectin and Pleiotrophin are differentially expressed in BM vessels (vascular heterogeneity). Levesque's group has shown that EC-specific E-selectin negatively regulates HSCs quiescence and self-renewal [8]. Chutes' group has shown that Pleiotrophin regulates HSCs chemotaxis and self-renewal [10]. These studies and ours strongly support the idea of differential vascular microenvironmental domains in the BM.

As was noted in Chapter 1, the BM niche paradigm has changed dramatically during the period of this project. Until 2008, the idea of a HSC-supportive endosteal niche and a differentiation and mobilization-supportive vascular niche was widely accepted [41-44] – despite the fact that the functional importance of the vascular niche for the hematopoietic recovery after myeloablation and anatomical studies were already suggesting a vascular niche for HSCs [25,28,29,37,45,46]. Since 2008, many other anatomic and functional studies suggested a vascular niche for HSCs, culminating in two reports published in 2013 that scrutinized the BM niches, with the identification of a vascular niche for HSCs and an osteoblastic niche for lymphoid-primed HPCs [10,30,47-51.] Our finding that

the BM vasculature is heterogeneous, and that exogenous treatments such as anti-Dll4 or chronic intermittent hypoxia change EC identity, will certainly be of great interest in the future. Indeed, we show that this shift in BM vascular identity also corresponds to a differential "angiocrine" gene expression. Further insight into this fine-tune modulation of BM vessels and its hematopoietic effects may reveal relevant features of hematopoiesis in both homeostasis and in malignancy [52].

Besides VE-Cadherin and CD31, we show that anti-Dll4 treatment modulates c-kit$^+$ vessels – a marker typically associated with HSPCs [53-57]. In fact, primary cultures of BM ECs revealed c-kit expression, however, its expression had never been assessed *in vivo* [58]. Previous reports demonstrated that BM arterioles express stem cell antigen (Sca)-1, another marker typically associated with HSPCs [47,59-61]. It is interesting to speculate the function of these stem cell markers in BM ECs. In 2012, a groundbreaking paper reported the existence of a small subpopulation of c-kit$^+$ vascular stem cells lining lung vessels [62]. Some of these cells were also Sca-1$^+$ [62]. Therefore, BM c-kit$^+$ ECs may be stem cells, and the increased c-kit$^+$ vessel coverage in anti-Dll4 treated mice may be related with an increased vascular stem cell percentage. This increase may be due to a promotion of vascular stem cell self-renewal to the detriment of differentiation. Or, do stem cells derived from the BM parenchyma (endothelial progenitor cells, EPCs) integrate blood vessels? Are BM ECs capable of dedifferentiation into vascular stem cells? If the ECs are capable of dedifferentiation, can they exit from the vessel and give rise to other cell types? In VECad-Cre-ER^{T2} adult mice, 3 weeks after induction, a subpopulation of BM cells with morphological features of hematopoietic cells is observed [63]. This observation may support the possibility of dedifferentiation or transdifferentiation of ECs, or, these cells may derive from VE-Cadherin$^+$ HSCs [64].

Thus far, we focused on the potential role of Dll4 in modulating the BM microenvironment and its consequences for hematopoiesis. However, when carefully analyzing the phenotype of VECad-Cre-ER^{T2}Dll4$^{lox/lox}$ mice, despite similar vascular changes than those observed in treated mice, the main hematopoietic lineages are not modulated as in mice treated with anti-Dll4 (Figures 2.8A, 5.1). Indeed, although *in vitro* and *in vivo* overexpres-

sion studies showed that microenvironment Dll4 regulates hematopoiesis [7,9,11-13,65-70], hematopoietic cells were also known to express Dll4 [65,71]. Thus, we searched for hematopoietic cell-specific responses to Dll4 blockade. Our data show that anti-Dll4 treatment of HSPCs increases myeloid differentiation in the absence of stromal cells (Figure 2.8C). Additionally, *in vitro* B cell differentiation assays reveal anti-Dll4 treatment of HSPCs decreases B-lymphopoiesis (Figure 5.2).

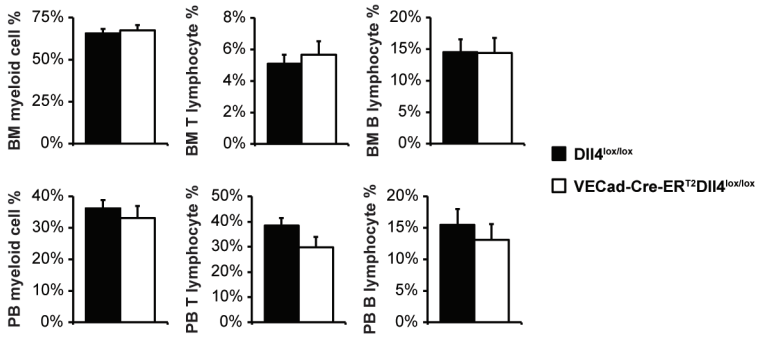


Figure 5.1. Endothelial-specific Dll4 blockade does not affect the main hematopoietic lineages.
Flow cytometric analysis of the percentage of myeloid cells (CD11b$^+$), T lymphocytes (CD3$^+$), and B lymphocytes (B220$^+$) in the BM and PB, revealing VECad-Cre-ER^{T2}Dll4$^{lox/lox}$ produce equivalent percentages of the main hematopoietic lineages.
Data are means ± s.e.m. *, p<0.05 ; n=11.

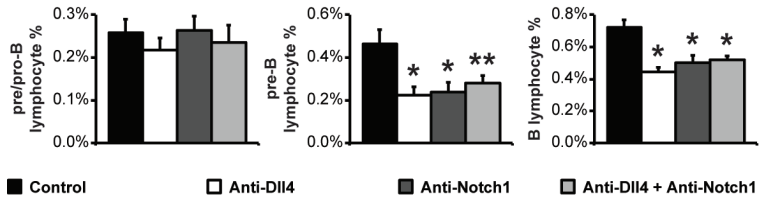


Figure 5.2. Anti-Dll4 blockade, through Notch1 inhibition, impairs B lymphopoiesis in a hematopoietic cell-specific manner.
Flow cytometric analysis of the percentage of pre/pro-B cells (CD34$^+$CD19$^+$), pre-B / immature B lymphocytes (CD34$^-$CD19$^+$), and B lymphocytes (CD19$^+$) throughout in vitro B cell differentiation reveals Dll4 blockade, through Notch1 signaling inhibition, blocks B lymphocyte differentiation at the pre-B lymphocyte stage. Hematopoietic stem/progenitor cells (105 cells/mL) were cocultured with immortalized stroma cells layers (S17 murine stroma cell line), and maintained in RPMI medium 3% FBS for 9 days. Anti-human antibodies (anti-Dll4, 50µg/mL MHD4-46, and anti-Notch1, 10µg/mL MHN1-519) were added to the coculture on the first day. Data are means ± s.e.m. *, p<0.05; **, p=0.06; n=4.

These data raised an important question with great therapeutic potential: if anti-Dll4 is targeting both the BM vessels and hematopoietic cells during hematopoietic recovery, can Dll4 blockade also directly target malignant hematopoietic cells? We had previously shown that transplantation of BM-derived Dll4$^{+/-}$ EPCs into leukemia-bearing mice, us-

ing an acute myeloid leukemia (AML) cell line, increases the tumor vascular density and reduces the tumor size [72]. Others had established an important relationship between endothelial-specific Dll4 levels and tumor dormancy in tumor xenografts of T-acute lymphoblastic leukemia (T-ALL) cell lines [73-75]. Indeed, Dll4 was recently shown to be overexpressed in human acute myeloid leukemia (AML) BM, which is associated with higher angiogenesis and poor prognosis [76,77]. All these studies suggested a potential role for the Dll4:Notch signaling pathway mediated by the microenvironment, however, Dll4 blockade does not only affect tumor angiogenesis, but also the tumor cells [78,79]. We screened several leukemia/lymphoma cell lines for Dll4 protein expression by Western Blotting, and found that all cell lines studied expressed Dll4 (Figure 3.1). Focusing on three cell lines representing different hematological diseases, AML (HL60), B-ALL (697), and T-ALL (MOLT-4), we measured the kinetics of spontaneous apoptosis and proliferation in *in vitro* assays where Notch signaling pathway was targeted. As shown in Figures 3.2 and 3.3, Dll4:Notch signaling pathway does not affect leukemia cell survival or growth, but Notch signaling inhibition promotes cell cycle arrest (Figure 3.2 and 3.3). We have not however assessed whether Dll4 modulates leukemia cell differentiation, which typically involves a cell cycle arrest in the G_0/G_1 phase [5]. This would be very interesting to assess, as we show that anti-Dll4 blockade favors myelopoiesis (Figure 2.8), and the Dll4-Notch pathway is required for several stages of hematopoiesis, promoting T cell differentiation at the expense of B cell proliferation [7,9,11-13]. In fact, co-culture of HL-60 cells with a stromal cell line overexpressing Dll4 promotes myeloid differentiation [14]. The exploitation of anti-Dll4 therapies as drivers of leukemia/lymphoma cell differentiation may have clinical relevance, as differentiation appears to be accompanied by differentiation-dependent apoptosis [5].

In a setting of BM transplant (BMT), we show that anti-Dll4 treatment of donor mice, post-myeloablation, improves hematopoietic recovery of recipient, lethally irradiated mice in BMT. We confirmed the involve-

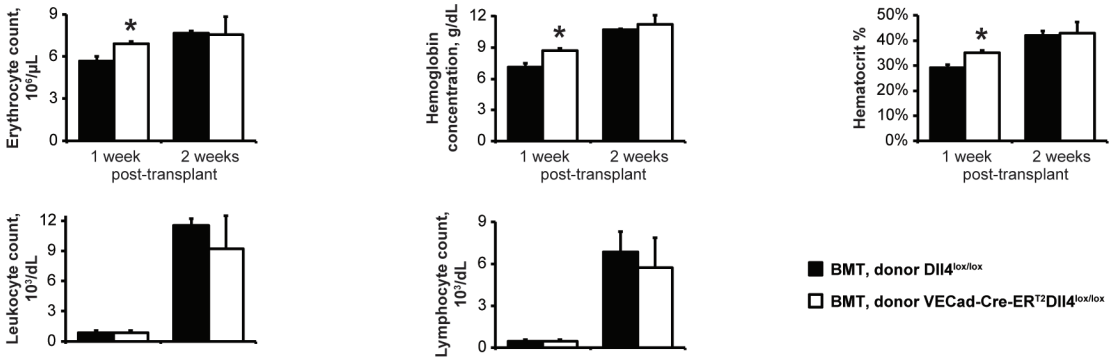


Figure 5.3. Endothelial-specific Dll4 blockade in donor BM improves hematopoietic recovery following transplantation into lethally irradiated recipients.
Erythrocyte, hemoglobin, hematocrit, leukocyte and lymphocyte quantifications were assessed by PB cell blood counts. Data shows donor VECad-Cre-ER^{T2}Dll4$^{lox/lox}$ mice induce faster recovery of erythrocytic parameters 1 week after transplantation.
Data are means ± s.e.m. *, p<0.05 ; n=11.

ment of endothelial-specific Dll4 in this modulation, with the observation that donor VECad-Cre-ER^{T2}Dll4$^{lox/lox}$ mice also improved hematopoietic recovery of recipient mice upon BMT (Figure 5.3). As discussed in Chapter 2, we suggest both "angiocrine" gene modulation (increased IGFbp2, IGFbp3 and Dll4) and increased CD31^{+} and VE-Cadherin^{+} BM vascular content upon anti-Dll4 treatment may explain this phenotype. However, it is perhaps more appealing to treat BMT recipients than donors – a healthy donor is more unlikely to be subjected to a therapy with potential secondary effects, such as the hepatic effects described in chapter 2 (Figure 2.5) and the hepatic, cardiac and vascular neoplasms reported during the progression of this study [20]. Therefore, we performed an experiment consisting in the treatment of BM recipients, at the time of injection of (untreated) donor BM. As always, BMT recipients were monitored daily and euthanized if they had evidence of severe weight loss and at least three of the following: dehydration (evaluated by skin tenting), lethargy and decreased movement, pale eyes, nasal and/or ocular discharge, or neuromuscular signs (incoordination or seizures). As shown in figure 5.4, unexpectedly,

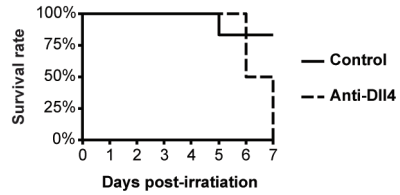


Figure 5.4. Recipient BMT mice treated with anti-Dll4 developed signs of severe morbidity by the first week after transplantation.
The Kaplan-Meier histogram depicts the survival rate of BMT recipients lethally irradiated (900rad) receiving 2.5*10^{6} BM mononuclear cells from untreated donors together with a single injection of 12.5g/kg anti-Dll4 (HMD4-2) or vehicle (PBS). n=6.

treated mice had to be euthanized at days 6 and 7 post-transplantation, while only one control was euthanized at day 5 post-irradiation for unknown reasons (Figure 5.4). These exploratory, albeit preliminary, experiments suggest further characterization of the importance of Dll4 for BM recovery following competitive transplants is needed.

A study published in 2013 by Maillard's group reports an improvement in graft-versus-host disease, the main complication of allogenic BMT, when recipient mice are treated with anti-Dll4 [22]. However, this was performed with an antibody developed by Genentech [16,22], while the one we used was produced by Hideo Yagita [27]. Post-transcriptional modifications such as glycosylation may underlie these differences. Notch receptor glycosylation is critical for modulation of Notch activity, changing the sensitivity of Notch receptors to Notch ligands [31,33,35]. Even though Notch ligands can be glycosylated [80], its importance for ligand function is still largely unknown. However, one could speculate that the different antibodies may bind to different sites of the Dll4 protein, or to glycosylated, unglycosylated, or in either state, thereby modulating its affinity to different Notch receptors – Notch1, 3 or 4 [73,81,82]. It will certainly be interesting to compare the antibodies currently tested in clinical trials and its potential clinical and side effects [20,83,84].

In conclusion, we reveal anti-Dll4 blockade following myeloablation modulates the BM vascular niche, by regulating the vascular identity and "angiocrine" gene expression, increases myelopoiesis and decreases lymphopoiesis both in a microenvironmental and hematopoietic cell-specific manner, and may improve hematopoietic recovery in a BMT setting (Figure 5.5).

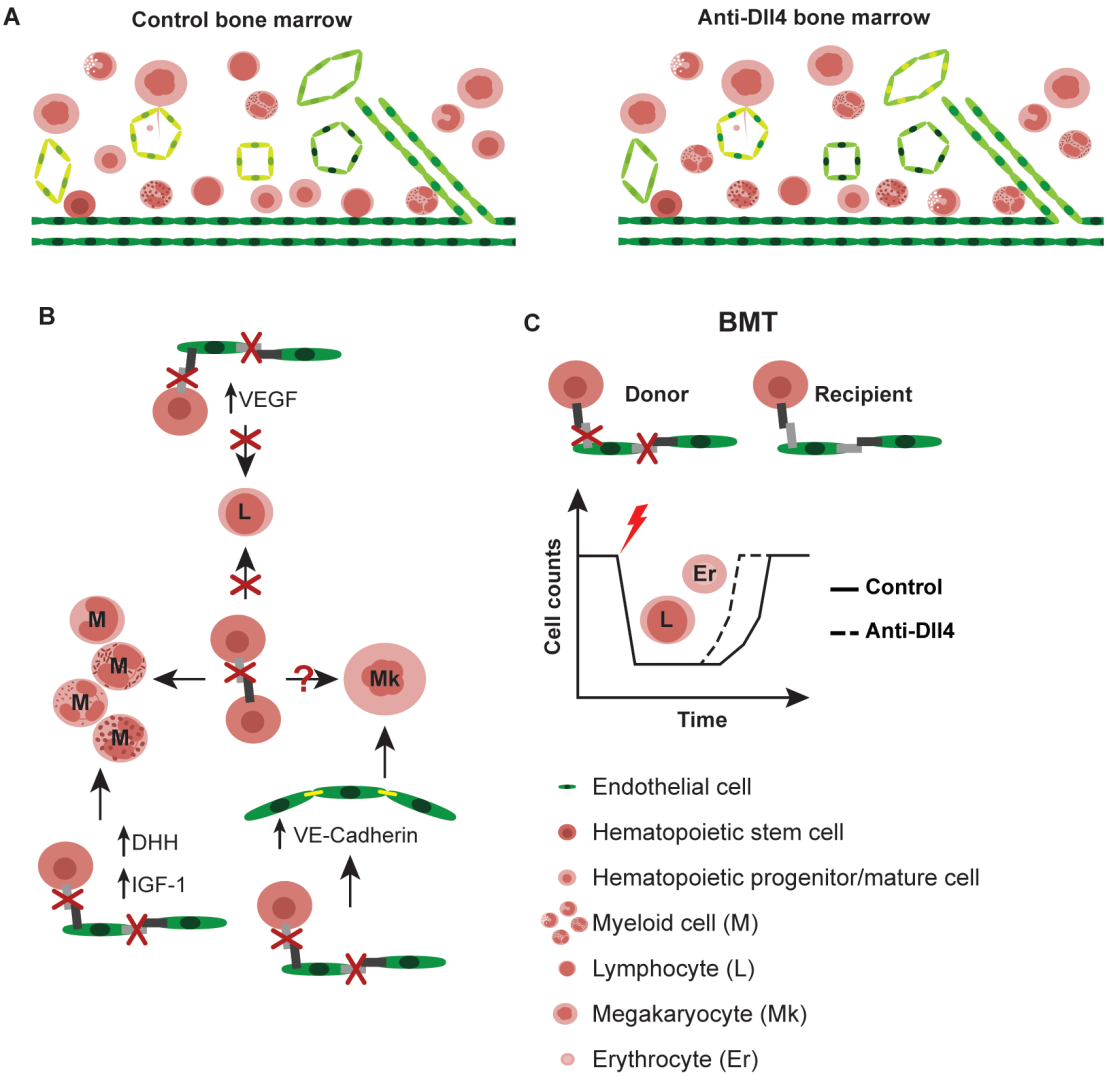


Figure 5.5. Representative figures of the major findings included in Chapter 2.
A. Model of the BM microenvironment upon Dll4 blockade. Delta-like 4 blockade modifies BM ECs identity, both phenotypically (by increasing the number of VE-Cadherin$^+$, CD31$^+$ and c-kit$^+$ vessels) and functionally (by modulating "angiocrine" genes expression, namely, increase of IGFbp2, IGFbp3, Angpt2, Dll4, DHH and VEGF-A, and decrease of FGF1 and CSF2). There is also an increase of megakaryocytes and myeloid cells both in BM and blood (not shown), and a decrease of lymphocyte in BM.
B. Model of the hematopoietic changes observed in anti-Dll4 treated mice following myeloablation. The myeloid compartment is expanded due to microenvironmental and hematopoietic cell-specific changes, whereas both microenvironmental and hematopoietic cell-specific modulation reduces BM lymphocyte content. The number of megakaryocytes is increased, due to microenvironmental changes (increased VE-Cadherin$^+$ vessels), and possibly to hematopoietic cell-specific changes (not tested, Poirault-Chassac 2010).
C. Model of the hematopoietic recovery after BMT, in which donor mice were treated with anti-Dll4. Both lymphocytes and erythrocytes recover faster when the donor mice were treated with anti-Dll4 (dashed line). Lightening bolt, lethal irradiation.

THE ROLE OF CHRONIC INTERMITTENT HYPOXIA, A MODEL FOR OBSTRUCTIVE SLEEP APNEA, IN THE BM MICROENVIRONMENT AND HEMATOPOIESIS

Hypoxia is a potent stimulator of angiogenesis [85-99], and modulates vasoconstriction/vasodilation [94,100,101]. Furthermore, environmental hypoxia modulates hematopoiesis, specifically, by increasing erythropoiesis and myelopoiesis, affecting thrombopoiesis biphasically, and promoting faster hematopoietic recovery following myeloablation [102-112]. Environmental hypoxia also modulates BM cellularity and HSPCs numbers [113-115], and *in vitro* studies suggest hypoxia maintains HSCs [116,117]. However, the role of environmental hypoxia in BM vasculature had not yet been assessed, nor its significance in terms of hematopoietic modulation.

To assess the hematopoietic modifications induced by the microenvironmental changes, and not the hypoxic cell-specific effects, we established a 3-day period of normoxia before collecting the data. This period was chosen according to the intravascular half-times of hematopoietic cells and platelets: rat circulating half-time of erythrocytes is about 19 days [118], of granulocytes 5-7 hours [119], of monocytes about 2-3 days [120,121], of lymphocytes 16 minutes [122], and of platelets 2 days [123]. We have chosen the 3-day period, since a longer period (to exclude the erythrocyte-specific hypoxic effect) could result in BM vascular remodeling.

Our preliminary results suggest CIH increases the overall $CD105^+$ BM vessels, and decreases VE-$Cadherin^+$ and smooth muscle actin $(SMA)^+$ vessels, without altering von Willebrand factor $(vWF)^+$ vascular density. This data suggest that unlike in anti-Dll4 blockade, hypoxia may increase overall BM vessel density by modulating specifically sinusoids, as pericyte-covered (SMA^+) vessels are decreased. However, this would have to be confirmed by using a combination of a sinusoidal marker such as VEGFR3 and an arteriole marker such as stem cell antigen-1 (Sca-1) [47,124]. As previously discussed, the assessment of different EC markers

may provide important information regarding BM microenvironmental domains. Additionally, our preliminary data suggest "angiocrine" gene modulation with increased expression of IL-6, Dll1, colony stimulating factor (CSF)2, CSF3, Smad3 and thrombopoietin (THPO) and a decreased expression of insulin growth factor (IGF)1, insulin growth factor binding protein (IGFbp)3, IGFbp5, angiopoietin 1 (Angpt1), stem cell factor (SCF) and N-Cadherin.

By increasing BM angiogenesis and erythropoiesis (Figure 4.4B,C, 4.2A), even after the normoxic period, CIH could be disrupting the normal oxygen homeostasis in the BM. Very simplistically, the oxygen tension (pO_2) in the BM is considered to be higher next to blood vessels and depends upon the oxygen consumed by each cell which intermediates a certain cell from a blood vessel [125,126]. However, hypoxia labeling using pimonidazole does not suggest increased hypoxia in the BM of rats subjected to CIH (Figure 4.4A) – in fact, previous reports demonstrate that 24 hours after the hypoxic period a tissue (skin) pO_2 is similar to that in control animals [97]. This suggests that our model allowed us to observe mainly the microenvironmental hematopoietic effects of hypoxia.

Our preliminary results suggest that CIH, followed by a normoxia period, increases erythropoiesis (Figure 4.2A), which is in concordance with a hypoxia-specific modulation of erythropoiesis, driven by erythropoietin during the hypoxic period [127-129], maintained through the 3-day normoxic period due to the long circulating half-time of erythrocytes [118] and eventually to an increased erythropoiesis driven by "angiocrine"-driven IL-6 [130,131]. There also seems to be an increase of circulating monocytes (Figure 4.2B), which may be due to an increased monocyte production driven by "angiocrine" expression of IL-6 (Figure 4.5). Chronic intermittent hypoxia may also promote thrombocytosis, which as discussed in Chapter 3 may be explained by an increased shear rate

and "angiocrine"-driven IL-6, CSF2 and THPO increase (Figure 4.2C, 4.5), and that, together with a normalization of megakaryopoiesis (see Chapter 3), may explain the reduced number of BM megakaryocytes and increased apoptotic megakaryocytes (Figure 4.4C,D).

These results may also reflect a modulation of hematopoiesis at the stem cell level. We intend to further pursue this project by observing the HSC compartment, which we have not yet done due to technical constraints: there are inter-species differences between HSCs markers, and the rat HSC markers are not yet scrutinized [56,132]. As mentioned in Chapter 1, HSCs are believed to reside in perivascular, hypoxic niches in the BM [6,45,50,51,133-138]. This has risen great attention to the role of hypoxia-inducible factors (HIFs) in hematopoiesis and bone marrow (BM) microenvironment [137,139-141]. However, even though HIF-α is typically considered to be degraded by the proteasome in most cell types in normoxia (triggered by the modification of HIF-α by prolyl hydroxylase (PHD) which uses oxygen as substrate [142] – see Figure 1.2), HIF-α was shown to be stable in normoxic conditions in both monocytes and leukemia cell lines [143,144]. Cellular iron, cobalt and ascorbate availability also mediate HIF-α-driven hypoxic response [145-149]. The reduction of cellular iron availability results in more stable HIF-α protein, as iron is the activating metal of PHD [150,151]. Cobaltous ions stabilize HIF-α by binding directly to HIF-α (and thus inhibiting the interaction between HIF-α and von Hippel-Lindau protein, VHL) [152], by binding factor inhibiting HIF-1 (FIH-1, thus disrupting its inhibitory effects upon HIF-α and VHL function) [153,154] or depleting intracellular ascorbate levels [155]. Finally, ascorbate is a cofactor for the function of PDH [156]. Thus, we do not consider the recent HIF-α studies fully comprise the possible role of low pO_2 in hematopoiesis, and believe our assessment is the best available tool: specific modulation of pO_2 in the BM is still technically unachievable, and the fraction of inspired oxygen is correlated with arterial pO_2 and BM pO_2 during acute modulations of atmospheric O_2 [157].

In conclusion, we used a clinically relevant approach to study the effects of hypoxia in BM microenvironment and its hematopoietic effects. Our preliminary results suggest that CIH increases the BM vascular content, modifies vessel type and identity and "angiocrine" gene expression, promotes erythropoiesis and myelopoiesis, deregulates megakaryopoiesis and promotes thrombocytosis (Figure 5.6).

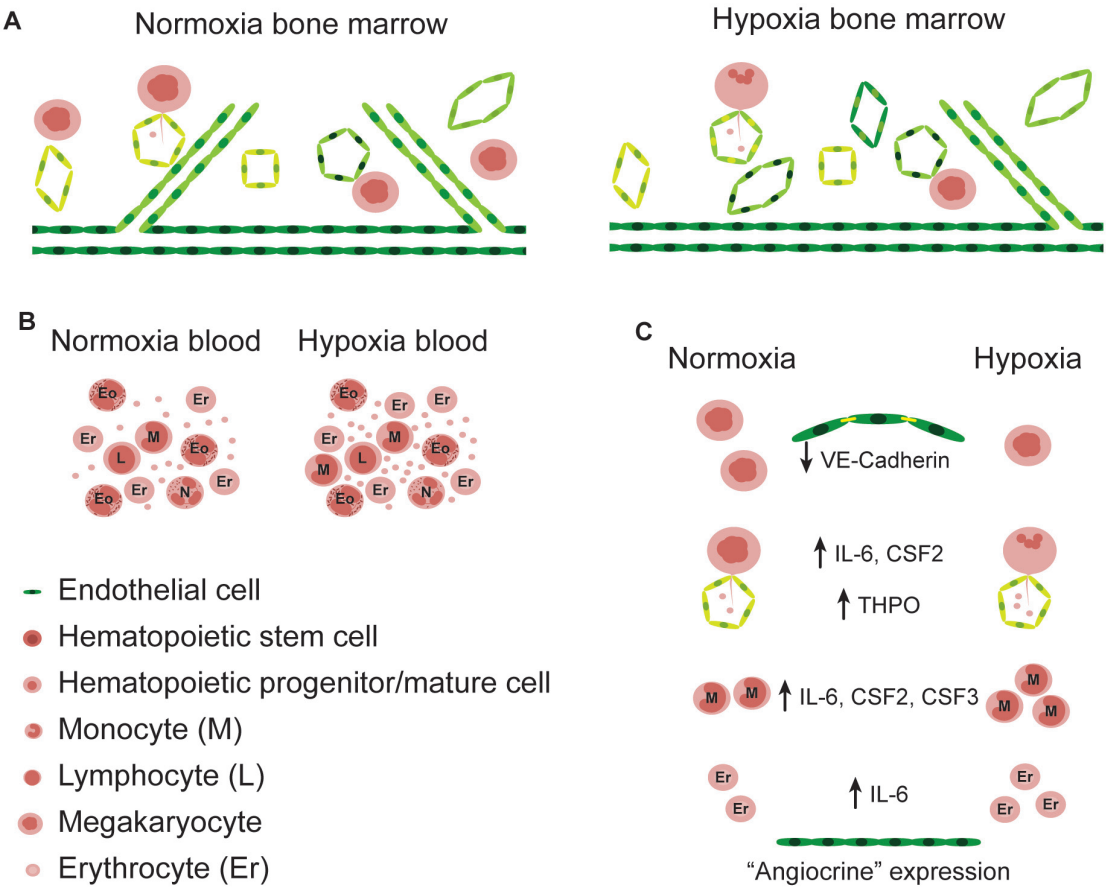


Figure 5.6. Representative figures of the major findings included in Chapter 3.
A. Model of the BM microenvironment upon chronic intermittent hypoxia treatment. Our preliminary data suggest hypoxia increases the overall vessel number ($CD105^+$) and modifies BM ECs identity, both phenotypically (by decreasing the number of VE-$Cadherin^+$ and pericyte-covered, SMA^+, vessels) and functionally (by modulating "angiocrine" genes expression, namely, increase of IL-6, Dll1, CSF2, CSF3, Smad3 and THPO, and decrease of IGF1, IGFbp3, IGFbp5, Angpt1, SCF and N-Cadherin expression). There is also a decrease of megakaryocytes and an increase in megakaryocyte apoptosis.
B. Model of the hematopoietic changes observed in the hypoxic animals. Our preliminary data suggests hypoxia increases peripheral blood erythrocytes, monocytes and platelets. (C) Proposed mechanisms that regulate the BM endothelial-driven hematopoietic modulation upon hypoxia treatment.

CONCLUDING REMARKS

In this Thesis, we provide evidence for a previously unappreciated BM vascular heterogeneity which is not only related to the vessel type, but also to microenvironmental domains that support different stages of hematopoiesis (Figure 5.7).

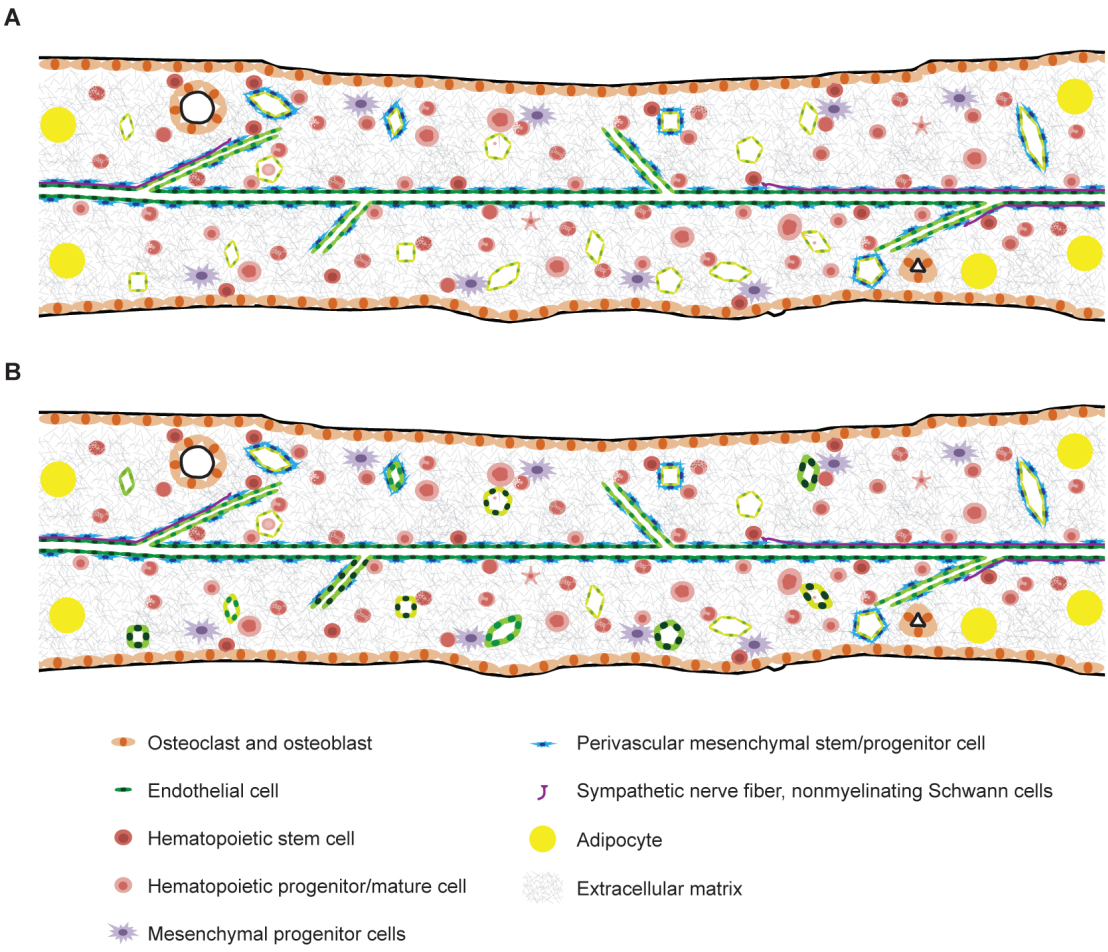


Figure 5.7. Models of the bone marrow microenvironment.
A. In 2008.
B. In 2012. We and others showed the heterogeneity of the BM vessels and started to reveal its significance in terms of hematopoietic modulation.

As noted in Chapter 1, the advance in scientific knowledge of BM function has been remarkable during these past four years, such that the role of the vascular compartment is now suggested to support not only hematopoietic cell adhesion, homing, differentiation and mobilization, but also to functionally maintain HSCs by regulating its self-renewal and proliferation [8,50,51,158]. The identification of BM microenvironmental domains created by heterogeneous vessels will allow a better understanding of the microenvironmental cues driving hematopoiesis in homeostasis and disease, which may originate the development of highly specific and efficient therapeutic drugs.

Specifically, we have explored the BM microenvironmental and hematopoietic changes upon anti-Dll4 treatment following myeloablation, important features for currently ongoing clinical trials [83,84], and in the setting of BM transplant (BMT). We found that anti-Dll4 treatment following myeloablation induces mild BM vascular and hematopoietic changes, and Dll4 may be an interesting therapeutic target in BMT. We further provide evidence for hepatic vascular abnormalities, which may have a decisive clinical impact for the use of anti-Dll4 treatment.

We also collected preliminary data regarding the modulation of the BM microenvironment and hematopoietic modulation in a model for obstructive sleep apnea, chronic intermittent hypoxia. Our data suggest that hypoxia modulates the BM microenvironment and hematopoiesis, and indicate that some of these changes are modulated by the BM ECs. This preliminary data not only indicates possible mechanisms for hematopoietic modulation in patients with obstructive sleep apnea, but also suggests a potential therapeutic approach for radio and chemotherapy and BMT, as increased BM vasculature has been correlated with faster hematopoietic recovery [28-30].

REFERENCES

1. Drummond, W. B. & Muir, R. On the Structure of the Bone-Marrow in Relation to Blood-Formation. *J Anat Physiol* 28, 125–141 (1893).

2. Dexter, T. M., Allen, T. D. & Lajtha, L. G. Conditions controlling the proliferation of haemopoietic stem cells in vitro. *J. Cell. Physiol.* 91, 335–344 (1977).

3. Schofield, R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* 4, 7–25 (1978).

4. Mazzarello, P., Calligaro, A. L. & Calligaro, A. Giulio Bizzozero: a pioneer of cell biology. *Nat Rev Mol Cell Biol* 2, 776–781 (2001).

5. Tsiftsoglou, A. S., Pappas, I. S. & Vizirianakis, I. S. Mechanisms involved in the induced differentiation of leukemia cells. *Pharmacol. Ther.* 100, 257–290 (2003).

6. Cooper, B. The origins of bone marrow as the seedbed of our blood: from antiquity to the time of Osler. *Proc (Bayl Univ Med Cent)* 24, 115–118 (2011).

7. Yan, X. Q. *et al.* A novel Notch ligand, Dll4, induces T-cell leukemia/lymphoma when overexpressed in mice by retroviral-mediated gene transfer. *Blood* 98, 3793–3799 (2001).

8. Winkler, I. G. *et al.* Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nature Medicine* (2012). doi:10.1038/nm.2969

9. Dorsch, M. *et al.* Ectopic expression of Delta4 impairs hematopoietic development and leads to lymphoproliferative disease. *Blood* 100, 2046–2055 (2002).

10. Himburg, H. A. *et al.* Pleiotrophin Regulates the Retention and Self-Renewal of Hematopoietic Stem Cells in the Bone Marrow Vascular Niche. *CellReports* (2012). doi:10.1016/j.celrep.2012.09.002

11. Koch, U. *et al.* Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *Journal of Experimental Medicine* 205, 2515–2523 (2008).

12. Hozumi, K. *et al.* Delta-like 4 is indispensable in thymic environment specific for T cell development. *Journal of Experimental Medicine* 205, 2507–2513 (2008).

13. Mohtashami, M. *et al.* Direct comparison of Dll1- and Dll4-mediated Notch activation levels shows differential lymphomyeloid lineage commitment outcomes. *The Journal of Immunology* 185, 867–876 (2010).

14. Sarmento, L. M. *et al.* Notch1 modulates timing of G1-S progression by inducing SKP2 transcription and p27 Kip1 degradation. *J. Exp. Med.* 202, 157–168 (2005).

15. Gale, N. W. *et al.* Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc. Natl. Acad. Sci. U.S.A.* 101, 15949–15954 (2004).

16. Ridgway, J. *et al.* Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature* 444, 1083–1087 (2006).

17. Noguera-Troise, I. *et al.* Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* 444, 1032–1037 (2006).

18. Lobov, I. B. *et al.* Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3219–3224 (2007).

19. Lee, S.-U. *et al.* LRF-mediated Dll4 repression in erythroblasts is necessary for hematopoietic stem cell maintenance. *Blood* 121, 918–929 (2013).

20. Yan, M. *et al.* Chronic DLL4 blockade induces vascular neoplasms. *Nature* 463, E1–E1 (2010).

21. Scehnet, J. S. *et al.* Inhibition of Dll4-mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion. *Blood* 109, 4753–4760 (2007).

22. Tran, I. T. *et al.* Blockade of individual Notch ligands and receptors controls graft-versus-host disease. *J. Clin. Invest.* (2013). doi:10.1172/JCI65477

23. Doan, C. A. *Contributions to Embryology*. XIV, 27–45 (Carnegie Institution of Washington, 1922).

24. Zamboni, L. & Pease, D. C. The vascular bed of red bone marrow. *J. Ultrastruct. Res.* 5, 65–85 (1961).

25. Kopp, H.-G., Avecilla, S. T., Hooper, A. T. & Rafii, S. The bone marrow vascular niche: home of HSC

differentiation and mobilization. *Physiology (Bethesda)* 20, 349–356 (2005).

26. Branemark, P. I. Experimental investigation of microcirculation in bone marrow. *Angiology* 12, 293–305 (1961).

27. Moriyama, Y. *et al.* Delta-like 1 is essential for the maintenance of marginal zone B cells in normal mice but not in autoimmune mice. *International Immunology* 20, 763–773 (2008).

28. Kopp, H.-G. *et al.* Tie2 activation contributes to hemangiogenic regeneration after myelosuppression. *Blood* 106, 505–513 (2005).

29. Chute, J. P. *et al.* Transplantation of vascular endothelial cells mediates the hematopoietic recovery and survival of lethally irradiated mice. *Blood* 109, 2365–2372 (2007).

30. Salter, A. B. *et al.* Endothelial progenitor cell infusion induces hematopoietic stem cell reconstitution in vivo. *Blood* 113, 2104–2107 (2009).

31. D'Souza, B., Miyamoto, A. & Weinmaster, G. The many facets of Notch ligands. *Oncogene* 27, 5148–5167 (2008).

32. van Buul, J. D. *et al.* Migration of human hematopoietic progenitor cells across bone marrow endothelium is regulated by vascular endothelial cadherin. *J. Immunol.* 168, 588–596 (2002).

33. Benedito, R. *et al.* The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* 137, 1124–1135 (2009).

34. Vaporciyan, A. A. *et al.* Involvement of platelet-endothelial cell adhesion molecule-1 in neutrophil recruitment in vivo. *Science* 262, 1580–1582 (1993).

35. Whiteman, P. *et al.* Molecular basis for jagged-1/serrate ligand recognition by the notch receptor. *J. Biol. Chem.* 288, 7305–7312 (2013).

36. Ross, E. A. *et al.* A Novel Role for PECAM-1 (CD31) in Regulating Haematopoietic Progenitor Cell Compartmentalization between the Peripheral Blood and Bone Marrow. *PLoS ONE* 3, e2338 (2008).

37. Avecilla, S. T. *et al.* Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nature Medicine* 10, 64–71 (2004).

38. Piali, L. *et al.* CD31/PECAM-1 is a ligand for alpha v beta 3 integrin involved in adhesion of leukocytes to endothelium. *The Journal of Cell Biology* 130, 451–460 (1995).

39. Wu, Y., Welte, T., Michaud, M. & Madri, J. A. PECAM-1: a multifaceted regulator of megakaryocytopoiesis. *Blood* 110, 851–859 (2007).

40. Dhanjal, T. S. *et al.* A novel role for PECAM-1 in megakaryocytokinesis and recovery of platelet counts in thrombocytopenic mice. *Blood* 109, 4237–4244 (2007).

41. Taichman, R. S. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. *Blood* 105, 2631–2639 (2005).

42. Suda, T., Arai, F. & Hirao, A. Hematopoietic stem cells and their niche. *Trends in Immunology* 26, 426–433 (2005).

43. Adams, G. B. & Scadden, D. T. The hematopoietic stem cell in its place. *Nat. Immunol.* 7, 333–337 (2006).

44. Wilson, A. *et al.* Dormant and self-renewing hematopoietic stem cells and their niches. *Ann. N. Y. Acad. Sci.* 1106, 64–75 (2007).

45. Kiel, M. J. *et al.* SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 121, 1109–1121 (2005).

46. Heissig, B. *et al.* Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* 109, 625–637 (2002).

47. Hooper, A. T. *et al.* Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. *Cell Stem Cell* 4, 263–274 (2009).

48. Butler, J. M. *et al.* Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell* 6, 251–264 (2010).

49. Ellis, S. L. *et al.* The relationship between bone, hemopoietic stem cells, and vasculature. *Blood* 118, 1516–1524 (2011).

50. Greenbaum, A. *et al.* CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* (2013). doi:10.1038/nature11926

51. Ding, L. & Morrison, S. J. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* (2013). doi:10.1038/nature11885

52. Lamorte, S., Remédio, L. & Dias, S. Communication between bone marrow niches in normal bone marrow function and during hemopathies progression. *Hematology Reviews* 1, (2009).

53. Okada, S. *et al.* Enrichment and characterization of murine hematopoietic stem cells that express c-kit molecule. *Blood* 78, 1706–1712 (1991).

54. Ikuta, K. & Weissman, I. L. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc. Natl. Acad. Sci. U.S.A.* 89, 1502–1506 (1992).

55. Orlic, D., Fischer, R., Nishikawa, S., Nienhuis, A. W. & Bodine, D. M. Purification and characterization of heterogeneous pluripotent hematopoietic stem cell populations expressing high levels of c-kit receptor. *Blood* 82, 762–770 (1993).

56. Osawa, M., Hanada, K., Hamada, H. & Nakauchi, H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* 273, 242–245 (1996).

57. Morrison, S. J., Wandycz, A. M., Hemmati, H. D., Wright, D. E. & Weissman, I. L. Identification of a lineage of multipotent hematopoietic progenitors. *Development* 124, 1929–1939 (1997).

58. Candal, F. J. *et al.* BMEC-1: a human bone marrow microvascular endothelial cell line with primary cell characteristics. *Microvasc. Res.* 52, 221–234 (1996).

59. Spangrude, G. J., Heimfeld, S. & Weissman, I. L. Purification and characterization of mouse hematopoietic stem cells. *Science* 241, 58–62 (1988).

60. Suzuki, N. *et al.* Combinatorial Gata2 and Sca1 expression defines hematopoietic stem cells in the bone marrow niche. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2202–2207 (2006).

61. Sugiyama, T., Kohara, H., Noda, M. & Nagasawa, T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 25, 977–988 (2006).

62. Fang, S., Wei, J., Pentinmikko, N., Leinonen, H. & Salven, P. Generation of functional blood vessels from a single c-kit+ adult vascular endothelial stem cell. *PLoS Biol.* 10, e1001407 (2012).

63. Monvoisin, A. *et al.* VE-cadherin-CreERT2 transgenic mouse: a model for inducible recombination in the endothelium. *Dev. Dyn.* 235, 3413–3422 (2006).

64. Arai, F. *et al.* Tie2/Angiopoietin-1 Signaling Regulates Hematopoietic Stem Cell Quiescence in the Bone Marrow Niche. *Cell* 118, 149–161 (2004).

65. Karanu, F. N. *et al.* Human homologues of Delta-1 and Delta-4 function as mitogenic regulators of primitive human hematopoietic cells. *Blood* 97, 1960–1967 (2001).

66. Lauret, E. *et al.* Membrane-bound delta-4 notch ligand reduces the proliferative activity of primitive human hematopoietic CD34+CD38low cells while maintaining their LTC-IC potential. *Leukemia* 18, 788–797 (2004).

67. Dando, J. S. *et al.* Notch/Delta4 Interaction in Human Embryonic Liver CD34 +CD38 –Cells: Positive Influence on BFU-E Production and LTC-IC Potential Maintenance. *Stem Cells* 23, 550–560 (2005).

68. Lahmar, M. *et al.* Distinct effects of the soluble versus membrane-bound forms of the notch ligand delta-4 on human $CD34^{+}CD38^{low}$ cell expansion and differentiation. *Stem Cells* 26, 621–629 (2008).

69. Poirault-Chassac, S. *et al.* Notch/Delta4 signaling inhibits human megakaryocytic terminal differentiation. *Blood* 116, 5670–5678 (2010).

70. Laranjeiro, R. *et al.* The Notch Ligand Delta-Like 4 Regulates Multiple Stages of Early Hemato-Vascular Development. *PLoS ONE* 7, e34553 (2012).

71. Chiaramonte, R. *et al.* A wide role for NOTCH1 signaling in acute leukemia. *Cancer Lett.* 219, 113–120 (2005).

72. Real, C. *et al.* Bone marrow-derived endothelial progenitors expressing Delta-like 4 (Dll4) regulate tumor angiogenesis. *PLoS ONE* 6, e18323 (2011).

73. Indraccolo, S. *et al.* Cross-talk between Tumor and Endothelial Cells Involving the Notch3-Dll4 Interaction Marks Escape from Tumor Dormancy. *Cancer Research* 69, 1314–1323 (2009).

74. Indraccolo, S., Minuzzo, S., Masiero, M. & Amadori, A. Ligand-driven activation of the notch pathway in T-ALL and solid tumors: why Not(ch)? *Cell Cycle* 9, 80–85 (2010).

75. Masiero, M. *et al.* Notch3-mediated regulation of MKP-1 levels promotes survival of T acute lymphoblastic leukemia cells. *Leukemia* 25, 588–598 (2011).

76. Zhang, J. *et al.* Prognostic impact of δ-like ligand 4 and Notch1 in acute myeloid leukemia. *Oncol. Rep.* 28, 1503–1511 (2012).

77. Zhang, J. *et al.* Cross-talk between Leukemic and Endothelial cells Promotes Angiogenesis by VEGF Activation of the Notch/Dll4 Pathway. *Carcinogenesis* (2012). doi:10.1093/carcin/bgs386

78. Hoey, T. *et al.* DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency. *Cell Stem Cell* 5, 168–177 (2009).

79. Yen, W.-C. *et al.* Anti-DLL4 Has Broad Spectrum Activity in Pancreatic Cancer Dependent on Targeting DLL4-Notch Signaling in Both Tumor and Vasculature Cells. *Clinical Cancer Research* 18, 5374–5386 (2012).

80. Panin, V. M. *et al.* Notch ligands are substrates for protein O-fucosyltransferase-1 and Fringe. *J. Biol. Chem.* 277, 29945–29952 (2002).

81. Rao, P. K. *et al.* Isolation and characterization of the notch ligand delta4. *Experimental Cell Research* 260, 379–386 (2000).

82. Shutter, J. R. *et al.* Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes & Development* 14, 1313–1318 (2000).

83. OncoMed Pharmaceuticals, Inc. A Phase 1 Dose Escalation Study of OMP-21M18 in Subjects With Solid Tumors. In: ClinicalTrials.gov. *Bethesda (MD): National Library of Medicine (US)* 1–3 (2013). at <http://clinicaltrials.gov/ct2/show/study/NCT00744562>

84. Regeneron PhamaceuticalsSanofi. A Multiple-Ascending-Dose Study of the Safety and Tolerability of REGN421(SAR153192) in Patients With Advanced Solid Malignancies. In: ClinicalTrials.gov. *Bethesda (MD): National Library of Medicine (US)* 1–3 (2013). at <http://clinicaltrials.gov/ct2/show/study/NCT00871559>

85. Opitz, E. Increased vascularization of the tissue due to acclimatization to high altitude and its significance for the oxygen transport. *Exp Med Surg* 9, 389–403 (1951).

86. Miller, A. T. & Hale, D. M. Increased vascularity of brain, heart, and skeletal muscle of polycythemic rats. *Am. J. Physiol.* 219, 702–704 (1970).

87. Knighton, D. R. *et al.* Oxygen tension regulates the expression of angiogenesis factor by macrophages. *Science* 221, 1283–1285 (1983).

88. Adair, T. H., Gay, W. J. & Montani, J. P. Growth regulation of the vascular system: evidence for a metabolic hypothesis. *Am. J. Physiol.* 259, R393–404 (1990).

89. LaManna, J. C., Vendel, L. M. & Farrell, R. M. Brain adaptation to chronic hypobaric hypoxia in rats. *J Appl Physiol* 72, 2238–2243 (1992).

90. Shweiki, D., Itin, A., Soffer, D. & Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359, 843–845 (1992).

91. Goldberg, M. A. & Schneider, T. J. Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *J. Biol. Chem.* 269, 4355–4359 (1994).

92. Harik, S. I., Hritz, M. A. & LaManna, J. C. Hypoxia-induced brain angiogenesis in the adult rat. *J. Physiol. (Lond.)* 485 (Pt 2), 525–530 (1995).

93. Harik, N. *et al.* Time-course and reversibility of the hypoxia-induced alterations in cerebral vascularity and cerebral capillary glucose transporter density. *Brain Res.* 737, 335–338 (1996).

94. Patt, S., Sampaolo, S., Théallier-Jankó, A., Tschairkin, I. & Cervós-Navarro, J. Cerebral angiogenesis

triggered by severe chronic hypoxia displays regional differences. *J. Cereb. Blood Flow Metab.* 17, 801–806 (1997).

95. Boero, J. A., Ascher, J., Arregui, A., Rovainen, C. & Woolsey, T. A. Increased brain capillaries in chronic hypoxia. *J Appl Physiol* 86, 1211–1219 (1999).

96. Howell, K., Preston, R. J. & McLoughlin, P. Chronic hypoxia causes angiogenesis in addition to remodelling in the adult rat pulmonary circulation. *J. Physiol. (Lond.)* 547, 133–145 (2003).

97. Saldívar, E., Cabrales, P., Tsai, A. G. & Intaglietta, M. Microcirculatory changes during chronic adaptation to hypoxia. *Am. J. Physiol. Heart Circ. Physiol.* 285, H2064–71 (2003).

98. Rofstad, E. K., Gaustad, J.-V., Egeland, T. A. M., Mathiesen, B. & Galappathi, K. Tumors exposed to acute cyclic hypoxic stress show enhanced angiogenesis, perfusion and metastatic dissemination. *Int. J. Cancer* 127, 1535–1546 (2010).

99. Gaustad, J.-V., Simonsen, T. G., Roa, A. M. A. & Rofstad, E. K. Tumors exposed to acute cyclic hypoxia show increased vessel density and delayed blood supply. *Microvasc. Res.* (2012). doi:10.1016/j.mvr.2012.11.002

100. Heistad, D. D. & Abboud, F. M. Dickinson W. Richards Lecture: Circulatory adjustments to hypoxia. *Circulation* 61, 463–470 (1980).

101. Voelkel, N. F. Mechanisms of hypoxic pulmonary vasoconstriction. *Am. Rev. Respir. Dis.* 133, 1186–1195 (1986).

102. Reissmann, K. R. Studies on the mechanism of erythropoietic stimulation in parabiotic rats during hypoxia. *Blood* 5, 372–380 (1950).

103. Bruce, W. R. & McCulloch, E. A. The Effect of Erythropoietic Stimulation on the Hemopoietic Colony-forming Cell of Mice. *Blood* 23, 216–232 (1964).

104. McGuire, M. & Bradford, A. Chronic intermittent hypoxia increases haematocrit and causes right ventricular hypertrophy in the rat. *Respir Physiol* 117, 53–58 (1999).

105. Coates, G., Nahmias, C. & Thind, A. Platelets and leukocytes in the lungs after acute hypobaric hypoxia. *J Appl Physiol* 55, 1536–1541 (1983).

106. Petursson, S. R. & Chervenick, P. A. Effects of hypoxia on megakaryocytopoiesis and granulopoiesis. *Eur. J. Haematol.* 39, 267–273 (1987).

107. Jackson, C. W. & Edwards, C. C. Biphasic thrombopoietic response to severe hypobaric hypoxia. *Br. J. Haematol.* 35, 233–244 (1977).

108. McDonald, T. P., Cottrell, M. & Clift, R. Effects of short-term hypoxia on platelet counts of mice. *Blood* 51, 165–175 (1978).

109. McDonald, T. P., Cottrell, M. B., Clift, R. E. & Jackson, C. W. Effects of hypoxia on megakaryocyte size and number of C3H and BALB/c mice. *Proc. Soc. Exp. Biol. Med.* 199, 287–290 (1992).

110. McDonald, T. P. *et al.* Comparison of platelet production in two strains of mice with different modal megakaryocyte DNA ploidies after exposure to hypoxia. *Exp. Hematol.* 20, 51–56 (1992).

111. Lebedeva, E. V., Yushkov, B. G. & Chereshnev, V. A. Megakaryocytopoiesis under hypoxic conditions. *Bull. Exp. Biol. Med.* 136, 554–556 (2003).

112. Beran, M. Hemopoietic recovery in posthypoxic mice: repopulation of CFUs and morphologically identifiable cells in the bone marrow and spleen. *Radiat. Res.* 53, 468–479 (1973).

113. Murphy, M. J. & Lord, B. I. Hematopoietic stem cell regulation. I. Acute effects of hypoxic-hypoxia on CFU kinetics. *Blood* 42, 81–87 (1973).

114. Lord, B. I. & Murphy, M. J. Hematopoietic stem cell regulation. II. Chronic effects of hypoxic-hypoxia on CFU kinetics. *Blood* 42, 89–98 (1973).

115. Loeffler, M., Herkenrath, P., Wichmann, H. E., Lord, B. I. & Murphy, M. J. The kinetics of hematopoietic stem cells during and after hypoxia. A model analysis. *Blut* 49, 427–439 (1984).

116. Cipolleschi, M. G., Sbarba, Dello, P. & Olivotto, M. The role of hypoxia in the maintenance of hematopoietic stem cells. *Blood* 82, 2031–2037 (1993).

117. Ivanovic, Z. *et al.* Incubation of murine bone marrow cells in hypoxia ensures the maintenance of marrow-repopulating ability together with the expansion of committed progenitors. *Br. J. Haematol.* 108, 424–429

(2000).

118. Goodman, J. W. & Smith, A. D. Erythrocyte life span in normal mice and in radiation bone marrow chimeras. *Am. J. Physiol.* 200, 764–770 (1961).

119. Gerecke, D., Schultze, B. & Maurer, W. Kinetics of neutrophilic granulocytes in the blood of rats. *Cell Tissue Kinet* 6, 369–378 (1973).

120. Whitelaw, D. M. The intravascular lifespan of monocytes. *Blood* 28, 455–464 (1966).

121. Volkman, A. & Collins, F. M. The cytokinetics of monocytosis in acute salmonella infection in the rat. *J. Exp. Med.* 139, 264–277 (1974).

122. Smith, M. E. & Ford, W. L. The recirculating lymphocyte pool of the rat: a systematic description of the migratory behaviour of recirculating lymphocytes. *Immunology* 49, 83–94 (1983).

123. Ashida, S. I. & Abiko, Y. Inhibition of platelet aggregation by a new agent, Ticlopidine. *Thromb. Haemost.* 40, 542–550 (1979).

124. Kopp, H.-G., Hooper, A. T., Avecilla, S. T. & Rafii, S. Functional heterogeneity of the bone marrow vascular niche. *Ann. N. Y. Acad. Sci.* 1176, 47–54 (2009).

125. Chow, D. C., Wenning, L. A., Miller, W. M. & Papoutsakis, E. T. Modeling pO(2) distributions in the bone marrow hematopoietic compartment. I. Krogh's model. *Biophys. J.* 81, 675–684 (2001).

126. Chow, D. C., Wenning, L. A., Miller, W. M. & Papoutsakis, E. T. Modeling pO(2) distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models. *Biophys. J.* 81, 685–696 (2001).

127. Eisensehr, I. & Noachtar, S. Haematological aspects of obstructive sleep apnoea. *Sleep Med Rev* 5, 207–221 (2001).

128. Jelkmann, W. Control of erythropoietin gene expression and its use in medicine. *Meth. Enzymol.* 435, 179–197 (2007).

129. Fukuda, M. N., Sasaki, H., Lopez, L. & Fukuda, M. Survival of recombinant erythropoietin in the circulation: the role of carbohydrates. *Blood* 73, 84–89 (1989).

130. Fletcher, E. C., Lesske, J., Qian, W., Miller, C. C. & Unger, T. Repetitive, episodic hypoxia causes diurnal elevation of blood pressure in rats. *Hypertension* 19, 555–561 (1992).

131. Suzuki, M. *et al.* Delta-4 expression on a stromal cell line is augmented by interleukin-6 via STAT3 activation. *Exp. Hematol.* 34, 1142–1149 (2006).

132. Butler, J. M. *et al.* Development of a vascular niche platform for expansion of repopulating human cord blood stem and progenitor cells. *Blood* 120, 1344–1347 (2012).

133. Allalunis, M. J., Chapman, J. D. & Turner, A. R. Identification of a hypoxic population of bone marrow cells. *Int. J. Radiat. Oncol. Biol. Phys.* 9, 227–232 (1983).

134. Parmar, K., Mauch, P., Vergilio, J.-A., Sackstein, R. & Down, J. D. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5431–5436 (2007).

135. Levesque, J.-P. *et al.* Hematopoietic Progenitor Cell Mobilization Results in Hypoxia with Increased Hypoxia-Inducible Transcription Factor-1α and Vascular Endothelial Growth Factor A in Bone Marrow. *Stem Cells* 25, 1954–1965 (2007).

136. Kubota, Y., Takubo, K. & Suda, T. Bone marrow long label-retaining cells reside in the sinusoidal hypoxic niche. *Biochemical and Biophysical Research Communications* 366, 335–339 (2008).

137. Takubo, K. *et al.* Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. *Cell Stem Cell* 7, 391–402 (2010).

138. Weigert, A. *et al.* HIF-1α is a negative regulator of plasmacytoid DC development in vitro and in vivo. *Blood* 120, 3001–3006 (2012).

139. Simsek, T. *et al.* The Distinct Metabolic Profile of Hematopoietic Stem Cells Reflects Their Location in a Hypoxic Niche. *Stem Cell* 7, 380–390 (2010).

140. Rehn, M. *et al.* Hypoxic induction of vascular endothelial growth factor regulates murine hematopoietic stem cell function in the low-oxygenic niche. *Blood* 118, 1534–1543 (2011).

141. Forristal, C. E. *et al.* Pharmacologic stabilization of HIF-1α increases hematopoietic stem cell quiescence in

vivo and accelerates blood recovery after severe irradiation. *Blood* 121, 759–769 (2013).

142. Semenza, G. L. Hypoxia-Inducible Factors in Physiology and Medicine. *Cell* 148, 399–408 (2012).

143. Cramer, T. *et al.* HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell* 112, 645–657 (2003).

144. Pedersen, M. *et al.* Stem cell factor induces HIF-1α at normoxia in hematopoietic cells. *Biochemical and Biophysical Research Communications* 377, 98–103 (2008).

145. Wang, G. L. & Semenza, G. L. Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood* 82, 3610–3615 (1993).

146. Goldwasser, E., Jacobson, L. O., Fried, W. & Plzak, L. Mechanism of the erythropoietic effect of cobalt. *Science* 125, 1085–1086 (1957).

147. Goldwasser, E., Jacobson, L. O., Fried, J. & Plzak, L. F. Studies on erythropoiesis. V. The effect of cobalt on the production of erythropoietin. *Blood* 13, 55–60 (1958).

148. Jaakkola, P. *et al.* Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* 292, 468–472 (2001).

149. Knowles, H. J., Raval, R. R., Harris, A. L. & Ratcliffe, P. J. Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. *Cancer Research* 63, 1764–1768 (2003).

150. Goldberg, M. A., Dunning, S. P. & Bunn, H. F. Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. *Science* 242, 1412–1415 (1988).

151. Dann, C. E., Bruick, R. K. & Deisenhofer, J. Structure of factor-inhibiting hypoxia-inducible factor 1: An asparaginyl hydroxylase involved in the hypoxic response pathway. *Proc. Natl. Acad. Sci. U.S.A.* 99, 15351–15356 (2002).

152. Yuan, Y. Cobalt Inhibits the Interaction between Hypoxia-inducible Factor-alpha and von Hippel-Lindau Protein by Direct Binding to Hypoxia-inducible Factor-alpha. *Journal of Biological Chemistry* 278, 15911–15916 (2003).

153. Mahon, P. C., Hirota, K. & Semenza, G. L. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes & Development* 15, 2675–2686 (2001).

154. Hirsilä, M. *et al.* Effect of desferrioxamine and metals on the hydroxylases in the oxygen sensing pathway. *The FASEB Journal* 19, 1308–1310 (2005).

155. Salnikow, K. *et al.* Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. *J. Biol. Chem.* 279, 40337–40344 (2004).

156. Veijola, J., Koivunen, P., Annunen, P., Pihlajaniemi, T. & Kivirikko, K. I. Cloning, baculovirus expression, and characterization of the alpha subunit of prolyl 4-hydroxylase from the nematode Caenorhabditis elegans. This alpha subunit forms an active alpha beta dimer with the human protein disulfide isomerase/beta subunit. *J. Biol. Chem.* 269, 26746–26753 (1994).

157. Eriksen, J., Tøndevold, E., Jansen, E. & Petersen, J. E. Relationships between oxygen and carbon dioxide tensions and acid-base balance in arterial blood and in medullary blood from long bones in dogs. *Acta Orthop Scand* 50, 519–525 (1979).

158. Kobayashi, H. *et al.* Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nature Cell Biology* 12, 1046–1056 (2010).

APPENDIX

COMMUNICATION BETWEEN BONE MARROW NICHES IN NORMAL BONE MARROW FUNCTION AND DURING HEMOPATHIES PROGRESSION

Review published in Lamorte S., Remédio L., Dias S. (2009) Communication between bone marrow niches in normal bone marrow function and during hemopathies progression. Hematology Reviews 1(2),e14.

Hematology Reviews 2009; volume 1:e14

Communication between bone marrow niches in normal bone marrow function and during hemopathies progression

Sara Lamorte,[1] Leonor Remédio,[1] Sergio Dias[1,2,3]

[1]Centro de Investigação de Patobiologia Molecular, CIPM, Instituto Português de Oncologia de Lisboa Francisco Gentil, E.P.E.; [2]CEDOC, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisboa, Portugal; [3]Instituto Gulbenkian Ciência, Oeiras, Portugal

Abstract

Hematopoietic stem cell (HSC) chemotaxis, adhesion, proliferation, quiescence and differentiation are regulated by interactions with bone marrow (BM) niches. Two niches have been identified in the adult BM: the endosteal (close to the bone) and the perivascular niche (close to blood vessels). A vast body of literature has revealed the molecular basis for the interaction of HSCs with the two niches. However, the signals that regulate the communication between the two niches have not been well defined. Taking in consideration several clinical and experimental arguments this review highlights the molecular cues, involved in the communication between the BM niches, which regulate the basic properties of HSCs in physiological and malignant conditions. As such, it aims at clarifying the most important advances in basic and clinical research focusing on the role of different factors in the regulation of the BM microenvironment.

Introduction

Hematopoietic stem cells reside in bone marrow niches, which regulate their fate

Hematopoietic stem cells (HSCs) are self-renewing cells which give rise to all types of mature blood cells. HSCs can be subdivided into long-term HSCs (LT-HSCs) and in short-term (ST-HSCs). LT-HSCs can give rise to all blood lineages and have unlimited self-renewal capacity. LT-HSCs produce ST-HSCs which are still multipotent but with limited self-renewal capacity. ST-HSCs differentiate further into lineage-committed progenitor cells which are responsible for the large-scale production of mature blood cells.[1]

The bone marrow (BM) is the major site of adult hemopoiesis, but, in pathological conditions, hemopoiesis can also occur in extramedullary sites like thymus, spleen and liver.

HSCs are localized in specialized microenvironments within hematopoietic tissues called niches.[2-6] Within the BM, two anatomical and functional niches have been proposed, the endosteal niche[7-10] and the perivascular niche.[11] It has been suggested that about 60% of bone-marrow HSCs are adjacent to perivascular niches and up to 20% of HSCs localize in the endosteal niches; the remaining HSCs are believed to be scattered throughout the BM.[11,12]

Endosteal niches, located at the inner bone surface, contain quiescent HSCs, characterized by a low proliferative rate; whereas activated HSCs, which undergo differentiation and ultimately mobilization to the peripheral circulation, are in close contact to sinusoids of the BM microvasculature in the perivascular niche[10,13-18] Endosteal niches may thus represent a reserve of HSCs, while perivascular niches connect HSCs to the blood stream.

The endosteal niche mainly comprises endosteal cells, osteoblasts and osteoclasts, while the perivascular niche contains mainly endothelial cells. Stromal cells, including reticular and mesenchymal cells, are common components of both niches. They are scattered throughout the trabecular space of the BM and surround the endothelial cells. As these cells are a component of both endosteal and vascular niches, they may serve as a cellular link between them.[15] The cellular components of the niches interact with each other to support HSC adhesion, quiescence, chemotaxis and, in the case of the vascular niche, differentiation.[10,14,16,17,19-21] Thus, the HSC properties and functional responses depend on specific interaction with BM niches (Table 1).

Chemotaxis

Bone marrow niches recruit hematopoietic stem cells

HSC chemotaxis towards the endosteal niche has been suggested to be mediated by osteoponin (Opn) and calcium ion concentration ($[Ca^{2+}]_o$).[25] Opn, a glycoprotein expressed on endosteal bone surface by osteoblasts, promotes HSC migration, as shown *in vivo* studies with $Opn^{-/-}$ mice. In these mice, there is a long-term engraftment defect after transplantation with wild-type $Lineage^-Sca^-1^+c\text{-}Kit^+$ cells and a compromised ability of the $Opn^{-/-}$ BM microenvironment to sustain hematopoiesis. These effects seem to be indirect, since there is no evidence, *in vitro*, of a chemotactic role for Opn on HSCs. Moreover, the high extracellular $[Ca^{2+}]^o$, maintained by the osteoclasts activity, promotes HSC localization to the endosteal niche, through calcium-sensing receptor (CaR): $CaR^{-/-}$ HSCs show a defect in the binding to collagenase I present at the bone endosteal surface.

Correspondence: Sergio Dias, CIPM, IPOLFG, R. Professor Lima Basto, 1099-023 Lisboa, Portugal.
E-mail: sergidias@ipolisboa.min-saude.pt

Key words: bone marrow niches, hematopoietic stem cell properties, molecular signals.

Acknowledgments: the authors would like to thank other members of the Angiogenesis Lab for their suggestions. Associação Portuguesa contra a Leucemia (APCL) and Liga Portuguesa Contra o Cancro (LPCC) fund our projects focusing on the bone marrow microenvironment. Sara Lamorte is funded by a fellowship from the University of Torino, Italy. Leonor Remédio is funded by an FCT (Portuguese Government) fellowship (SFRH/BD/43531/2008).

Received for publication: 26 May 2009.
Accepted for publication: 27 July 2009.

Hematology Reviews 2009; 1:e14
doi:10.4081/hr.2009.e14

Migration of HSCs from the endosteal to the perivascular niche is regulated by c-kit/Stem Cell Factor (SCF); CXC chemokine receptor 4 (CXCR4)/stromal-cell derived factor-1 (SDF-1) and granulocyte colony-stimulating factor (G-CSF), pathways.[7,22-24,42] Endothelial cells and reticular cells have been shown to produce SDF-1, generating a gradient from the perivascular to the endosteal niche, which may thus promote HSCs migration, since CXCR4 is expressed on HSC.[22-24] Mobilization of HSCs from the endosteal to the vascular niche is essential for hemopoietic recovery following myeloablation. In this case, the soluble form of membrane stem cell factor (sSCF), released from osteoblasts after cleavage by SDF-1-induced matrix metalloproteinase-9, promotes HSC homing to the perivascular niche by interacting with its receptor c-Kit.[7,22] G-CSF, produced by osteoblasts, promotes the mobilization of HSCs into the peripheral blood by up-regulating CXCR4 expression on HSCs and decreasing SDF-1 expression in the BM. G-CSF, in fact, induces the expression of proteolytic enzymes such as elastase, cathepsin G, MMP-2, and MMP-9, which cleave SDF-1.[42,43]

Adhesion

Bone marrow niche promotes hematopoietic stem cells adhesion

HSC adhesion to the endosteal niche is regulated by different molecular interactions including N-cadherin/β-catenin; Tie-2/Angio-

APPENDIX

poietin-1 (Ang-1); Osteopontin (Opn)/ß1 integrin; Annexin II (Anxa2)/Anxa2 receptor (Anxa2r) and CaR-collagen I pathways.[10,25,28,33] The asymmetrical distribuition of N-cadherin/β-catenin on the cell surface of HSCs and osteoblasts, respectively, and, in particular, the localization of these molecules at the site of interaction of LT-HSC with spindle-shaped N-cadherin^{+}CD45^{-} osteoblastic (SNO) cells, suggested a role for N-cadherin/ß-catenin in HSCs adhesion on the endosteal niches.[10] Studies performed by Kiel and collaborators failed to show significant numbers of N-cadherin expressing HSCs, questioning whether HSC adhesion to osteoblasts is mediated by N-cadherin.[44]

Tie2, a receptor tyrosine kinase expressed by a small fraction of BM cells highly enriched for HSC activity in adult murine BM, binds its ligand, Ang-1, expressed by osteoblasts at the surface of trabecular bone.[33] Regarding Opn, its expression is restricted to the endosteal bone surface and contributes to HSCs adhesion to the endosteal region via ß1 integrin expressed by HSC.[25] Osteoblasts also express high levels of Anxa2, a calcium-dependent phospholipid-binding protein, and it has been shown, both *in vitro* and *in vivo*, that Anxa2 regulates HSCs homing and binding to the endosteal niche, through the binding to its ligand Anxa2r.[28]

Adhesion of HSCs to the perivascular niche is mediated by $\alpha_4\beta_1$ integrin/vascular cell adhesion molecule1 (VCAM1) and α_4/E-selectin interaction.[26,27,45] $\alpha_4\beta_1$ integrins, expressed by HSCs, interact with VCAM-1, constitutively expressed on BM endothelial cells.[26] Since inactivation of E-selectin and α_4 integrin reduces drastically hematopoietic progenitor and stem cell (HPSC) homing into lethally irradiated mice, it has been proposed that E-selectin ligands and α_4 integrin cooperate in HSC adhesion to perivascular niches.[27]

Proliferation versus quiescence

Endosteal niches promote HSC quiescence

The balance between HSC proliferation and quiescence is likewise regulated by several pathways. In the endosteal niche several interactions, involved in the maintenance of HSC quiescence, have been identified: Tie-2/Angiopoietin-1 (Ang-1); thrombopoietin (THPO)/MPL; Opn/OpnR; parathyroid hormone (PTH)/PTH receptor (PTHr) and Notch1/Jagged1.[14,25,29,33-35,37,46] Tie2, which is expressed by SP-HSCs, binds Ang-1^{+} expressed on osteoblasts and induces HSC quiescence.[33,34] LT-HSCs expressing MPL, the THPO receptor, are closely associated with THPO-producing osteoblasts. The THPO/MPL pathway is involved in HSC quiescence through activation of genes coding for negative regulators of cell cycle, such as *p12*^{Cip1} and *p57*^{Kip2}, and inhibition of positive regulators, such as *c-myc*.[46] This pathway is also involved in promoting HSCs proliferation in the perivascular niche.[30-32] Thus, THPO/MPL pathway exerts distinct functions on HSC, depending on cell localization. Opn/OpnR, instead, contributes to the maintenance of HSC quiescence either by inhibiting, in a dose-dependent manner, the entry into cell cycle and/or by reducing cell apoptosis.[25,29] A mouse genetic model, in which the gene *PTHr* is constitutively active in osteoblasts, showed an increase in HSCs along with osteoblasts. Moreover, there was high expression of Notch I ligand, Jagged, on osteoblasts, suggesting that the PTH/PTHr pathway can promote HSC proliferation through activation of Notch.[14,37] Several gain- and loss-of-function experiments of Notch target genes and ligands have suggested a role for Notch in HSC quiescence and self-renewal.[35] However, recently Maillard *et al.* have demonstrated rather conclusively that inactivation of the Notch pathway in HSCs does not interfere with their self-renewal; transplantation of hematopoietic progenitors with inhibited Notch signaling induced stable long-term reconstitution of irradiated hosts and a normal frequency of progenitor fractions enriched for LT-HSCs.[36]

Table 1. HSC properties are regulated by molecular cues conveyed by the bone marrow endosteal and vascular niches.

HSC properties	Molecular interactions HSC-NICHE		
	Endosteal niche		Perivascular niche
Chemotaxis	Opn[25] CaR/Ca^{2} c-Kit/SCF[22]	CXCR4/SDF-1[22-24]	G-CSF[22]
Adhesion	N-cadherin/ß-catenin[10] Tie2/Ang1[10] ß$_1$ integrin/Opn[25] Anxa2/Anxa2r [28] CaR/Collagen I[25]		$\alpha_4\beta_1$ integrin/VCAM1[26] α4 integrins/E-selectin[27]
Quiescence / proliferation	Tie2/Ang1[33,34] Notch1/Jagged1[35,36] Opn[25,29] PTH/PTHr[14,37]	MPL/THPO [29-32]	wnt/β catenin[47,48]
Differentiation		FGF-4[13] SDF-1/CXCR4[38] $\alpha_4\beta_1$ integrin/VCAM[13] VE-Cadherin[13] Notch1/Delta[39-41]	

Perivascular niches promote hematopoietic stem cells proliferation and self-renewal

In the vascular niche, HSC proliferation is associated with (THPO)/c-mpl and Wnt/β catenin pathway. THPO is expressed on BM stromal cells and acts synergistically with erythropoietin to promote erythroid progenitors and megakaryocytes proliferation. THPO stimulates *c-myc* mRNA expression through a PI3K- and MAPK-dependent pathway, thereby promoting HSC proliferation.[30-32] Wnt proteins are expressed by BM stromal cell and exposure to Wnt was shown to stimulate proliferation and self-renewal of HSCs *in vitro*.[47,48]

Differentiation

Perivascular niches mediate hematopoietic stem cell differentiation

Differentiation of HSCs occurs only in the perivascular niches and is mediated by FGF-4; SDF-1; VCAM-1/α_4ß ; VE-cadherin and Notch1 pathway.[13,38,39,49] SDF-1 is necessary for myelopoiesis and B-lymphopoiesis, as shown by the severe reduction of B-lymphopoiesis and lack of BM myelopoiesis in CXCR4- and SDF-1 deficient mice.[49] SDF-1 and FGF-4 promote megakaryocyte maturation and platelet production: FGF-4 supports the adhesion of megakaryocytes to sinusoidal BM endothelial cells (BMECs), thereby enhancing their survival and maturation, while SDF-1 augments platelet production by promoting their migration across BMECs.[13,38] VCAM-1 enhances the interaction of $\alpha_4\beta_1$ integrin $^{+}$ megakaryocytes with BMECs. VE-cadherin is essential for VCAM-1 expression in BMECs, which in turn is required for FGF-4 mediated adhesion and SDF-1-induced transendothelial migration of megakaryocytes. Neutralizing antibodies to VE-cadherin decrease the localization of megakaryocytes to the vascular niche and disrupt megakaryocyte maturation and thrombopoiesis.[13] Notch1 seems to provide a key reg-

ulatory signal in determining T- versus B-lymphoid lineage commitment. Mice transplanted with BM, transfected for retroviruses encoding a constitutively active form of Notch1, three weeks after transplantation showed immature $CD4^+CD8^+$ T cells in the BM and a block in early B-cell lymphopoiesis.[39] Notch1 activation seems to be driven by Delta-1-expressing stromal cells.[40,41]

Hemopathies require the support of aberrant bone marrow niches

Several hemopathies are characterized by a pre-malignant phase that progresses to a malignant phase. The molecular basis of this progression remains poorly understood. The data in the literature suggest the likelihood of such progression is very low and a malignant clone can remain "stable" for years. Moreover, in several diseases both phases are characterized by virtually the same genetic changes.[50,51] Taking in consideration these aspects, it is legitimate to speculate that the genetic changes are necessary for the immortalization of a malignant clone but insufficient to promote the progression to a malignant phase. So other factors must take part in the progression.

The role of the hematopoietic BM microenvironment in malignant progression has been studied extensively and its importance was well illustrated in recent, *in vivo*, studies. Widespread inactivation of retinoblastoma protein (Rb) resulted in myeloproliferative disease, characterized by extramedullary hemopoiesis and increased mobilization and differentiation of HSCs from the BM. The phenotype was not recapitulated upon inactivation of Rb in HSCs maintained in wild-type environment.[52,53] Moreover, *Mx-Cre*$^+$*Pten*$^{\gamma/\Delta}$Δmice develop rapid and aggressive myeloproliferation that progressed to leukemia in 4-5 weeks post deletion. When *Pten* deletion was active in the context of a wild-type BM microenvironment, phenotypic and functional HSCs were lost without evidence of myeloproliferation or transformation.[54,55] Finally, BM from wild-type mice transplanted into mice with a deficient retinoic acid receptor γ (RARγ) microenvironment rapidly develop myeloproliferative syndromes (MPS).[56] These results strongly support the notion that the progression of the hemopathies is not entirely cell autonomous but depends on interactions between malignant cells and the BM microenvironment (BMM). As described above, BM niches support HSC properties such as adhesion, quiescence, chemotaxis and differentiation, and regulate the balance between self-renewal and differentiation. The idea outlined in this review is that alteration of the two BM niches, triggered by the aberrant expression of key molecules or cellular cues between the endosteal and the perivascular niche, impairs HSC responses, contributing to the progression of hemopathies. In chronic myeloid leukemia (CML), myelodysplastic syndromes (MDS) and multiple myeloma (MM) circulating endothelial cells (CECs), mobilized from the BM, share chromosomal aberrations with the malignant hematopoietic cells.[57-59] These malignant CECs suggest the presence of aberrant niches in the BMM. Moreover, in B-cell lymphomas, identical genetic aberration could be found both in malignant cells and in the microvascular BM endothelial cells.[60,61]

Irradiation and chemotherapy can change the BMM inducing hematopoietic and endothelial injury and allowing cells, proteins and cytokines to move between the vascular and endosteal niches.[62] Radiation-induced injury can also contribute to cell damage in the microenvironment in an indirect way, as a consequence of an inflammatory-type response.[63] Moreover, it has been shown that ionizing irradiation results in altered osteoblast differentiation ability of BM mesenchymal stem cells, destruction of the endosteal niche and consequently hematopoietic injury.[64] Another possibility is that malignant cells through direct and indirect signaling can modify the features of the vascular niche. For example, factors produced by acute lymphoblastic leukemia (ALL) cells can induce proliferation, migration and morphogenesis of human BM vascular endothelial cells.[65-67] The tumor-derived factor VEGF and tumor necrosis factor-α (TNF-α) produced in the tumor microenvironment have been shown to modify the phenotype of endothelial cells inhibiting ICAM-1 and VCAM-1 clustering on endothelial surfaces with implications for immune-cell trafficking.[68] Moreover, our own data suggests TNF-α is crucial for the onset and also for the progression of BM dysfunction, such as in MDS (*Cachaco et al., 2009, unpublished data*).

The possible mechanisms by which aberrant BM niches modify HSCs properties are discussed.

Migration/chemotaxis

Aberrant niches may promote recruitment of malignant hematopoietic stem cells

The perivascular niche expresses unique combinations of cell adhesion molecules and/or chemokines capable of attracting malignant HSCs. For example, it has been shown, *in vitro* and *in vivo*, that E-selectin and SDF-1 are expressed in vascular "hot spots" corresponding to the regions that attract leukemic cells.[69-71] Distruption of the interaction between SDF-1 and its receptor CXCR4 inhibits the homing of Nalm-6 cells, an acute lymphoblastic leukemia cell line, to the vascular niche.[71] These observations raise the possibility that E-selectin and /or SDF-1 can regulate malignant cell homing. Moreover, BM endothelial and stromal cells seem involved in the migration of ALL cells beneath BM fibroblast layers: both cell types produce SDF-1, thereby enhancing the adhesion molecules involved in the migration and homing of these cells to the BM.[72,73]

Adhesion

Aberrant niches mediate cell-adhesion-mediated drug resistance (CAM-DR)

It has been demonstrated, *in vitro* and *in vivo*, that cell-cell adhesion between hematopoietic cells and components of the BM niches, such as stromal cells, is involved in drug resistance in AML.[74,75] AML resistance to chemotherapy seems to be promoted by the adhesion-dependent secretion of WNT antagonists by osteoblasts.[76] CAM-DR is mediated by integrins α_4 and β1, as shown in MM, CML and AML cell lines.[77-79] Direct correlation has been found between the expression of integrins that mediate adhesion to FN and drug resistance.

Coculture of ALL cells lines with BM stroma cells (BMSCs) resulted in reduced apoptosis induced by etoposide. In this stroma model, drug resistance required direct cell-cell contact, since it could not be conferred by the addition of stromal conditioned media.[80] Moreover, the presence of BMSCs during treatment of myeloma cell lines significantly decreases the apoptosis during exposition of mitoxantrone, an inhibitor of topoisomerase II.[81] Notch-1 signaling seems to be involved in protection of MM from drug-induced apoptosis: overexpression of Notch-1 in Notch-1(-) myeloma cells up-regulated p21 and resulted in protection from drug-induced apoptosis.[82] BM niches may provide a survival advantage for malignant cells following initial drug exposure and facilitate the acquisition of acquired drug resistance, determining disease relapse following chemotherapy.

Aberrant niches show impaired adhesive capacity, leading to a loss of quiescence and consequently to expansion of malignant hematopoietic stem cells

It has been hypothesized that HSC mobilization results from impaired adhesion to BM niches, allowing their migration into the peripheral blood, spleen and other extramedullary sites. This could explain the increase in circulating $CD34^+$ cells reported in primary myelofibrosis (PMF) patients.[83,84]

The impaired adhesion could be explained by several mechanisms.

Altered expression of membrane adhesion molecules and integrins. For example, HSCs of CML patients have reduced adhesion molecules expression including L-selectin, CD44 and N-cadherin. This decrease correlates with, *in vitro*, reduced adhesive capacity of HSCs from CML patients.[85]

A disruption of CXCR4/SDF-1 axis. In idiopathic myelofibrosis (IM) the constitutive mobilization of $CD34^+$ cells could be the conse-

quence of the creation of a proteolytic microenvironment within the BMM. It has been shown that malignant cells and the BMM produce metalloproteinase.[86-88] Thus, the increased production of metalloproteinase-9 might disrupt adhesive interaction between CD34+ HSCs and BM niches through degradation of SDF-1 or cleavage of its receptor CXCR4, leading to the release of the HSCs into the peripheral blood.[22,89]

Proliferation *vs.* quiescence

Aberrant niches determine an imbalance between proliferation and quiescence, accelerating the onset and progression of malignancy

BM cells display a different set of adhesion molecules, extracellular matrix elements, growth factors and chemokines. Spleen fibroblasts isolated from PMF patients, in contrast to primary fibroblasts purified from the spleen of healthy subjects, are able to support the proliferation of autologous patient CD34+ cells, but not that of their normal counterparts.[90] Moreover, it has been shown that somatic mutations that occur in BM stromal cells, such as p53 mutations, render these cells supportive of ALL growth.[81] Finally, aberrant vascular niches produce several factors, such as VEGF; IL-6; granulocyte-macrophage and granulocyte colony-stimulating factors, that are able to support malignant hemopoiesis.[91-93] For example, it has been shown that coculture of AML cells with microvascular endothelial cells increases proliferation and inhibits apoptosis of AML cells.[93]

Providing self-renewing and proliferative cues to malignant HSCs. ALL stromal cells regulate self-renewal and proliferation of a Philadelphia-chromosome positive (Ph+)/VE-cadherin+ subpopulation of leukemia cells by promoting the expression of VE-cadherin, stabilizing β catenin and up-regulating BCR-abl transcripts.[94] This way, due to the stromal support, malignant cells circumvent the requirement of exogenous Wnt signaling for self-renewal. Human MM cells also become independent of the IL-6/gp130/STAT3 survival pathway when cocultured in the presence of BMSCs.[95] This evidence confirms the idea that BMSCs can provide alternative survival and proliferative signals to BM malignant cells.

Angiogenesis, the branching of new microvessels from pre-existent blood vessels, is kept at set point in which there is a balance between pro- and anti-angiogenic molecules. The angiogenic switch, unbalanced set point in favor of pro-angiogenic molecules, favors the production of new microvessels.[96] Increased angiogenesis has been described in a number of hemopathies.[97-101] The extent of BM neo-vessel formation correlates also with patient prognosis and these hemopathies are sensitive to anti-VEGF and VEGF receptor treatments.[102-105] The expanded BM endothelium may support malignant HSC growth by protecting them from chemotherapy-induced apoptosis and/or promoting their proliferation in a paracrine way through the release of factors such as G-CSF, IL-10, IL-6 and vascular endothelial growth factor-C (VEGF-C).[106,107]

Aberrant vascular niches can induce quiescence in malignant cells playing a role in tumor maintenance

Adhesion of malignant HSCs to BMSCs may induce quiescence by inhibiting cell proliferation. For example, Notch-1 activation in MM cells, after incubation on BMSCs, results in the accumulation of the cells in G0/G1 phase of cell cycle.[82,108] Aberrant niches may thus contribute to the maintenance of a malignant pool of HSCs.

Differentiation

Aberrant niches can induce malignant transformation of normal hematopoietic stem cells

The donor cell leukemia (DCL), a hemopathy following hemopoietic cell transplation, is apparently the result of malignant transformation of normal donor hematopoietic cells in the transplant recipient.[109] One of the hypotheses is that the host microenvironment in which the original malignancy developed may trigger malignant transformations in donor cells, favored by the immunocompromised status after transplantation and by perturbation of the host BMM following multiple rounds of chemotherapy.

Studies in *Drosophila Melanogaster,* by Kai *et al.,* suggest that a vacant niche can engage ectopic cells, normal hematopoietic and non-hematopoietic cells, with a resultant change in phenotype. Depending on the specific system, it seems that non-stem cells can acquire either a more proliferative phenotype or revert to a stem cell-like condition. These findings strongly support the possibility that BM niches can contribute to hemopathies, inducing aberrant transformation of normal cells, including HSCs.[110,111]

Bone marrow niches as therapeutic target

Based on the idea that the BMM has a relevant role in the progression of hemopathies, novel therapeutic approaches are being developed to revert the malignant phenotype by targeting environmental cues. The strategies used until now can be summarized into three categories.

The first strategy is to modify the niche itself. For example, Ballen and colleagues have tested the hypothesis to use parathyroid hormone (PTH) to augment the engraftment efficiency of cord blood transplant, modifying the receptivity of the endosteal niche.[112] PTH, acting also on the perivascular niche, can be used for the treatment of ischemic vascular disease.[113] Moreover, it has recently been shown that pharmacological use of PTH increases the number of HSCs mobilized into the peripheral blood for stem cell harvests, protects stem cells from repeated exposure to cytotoxic chemotherapy and expands stem cells in transplant recipients.[114]

The second strategy is to abrogate the interaction between malignant HSCs and BM niches, by blocking their physical binding or the growth factors secreted by the BMM. As described before, the chemokine axis SDF-1/CXCR4 is involved in the retention of HSCs within the BM. Thus, destruction of this interaction allows the mobilization of HSCs from the BM to the peripheral blood. This approach has been established clinically using G-CSF or antibody against CXCR4.[115-117] The combination of both result in an enhancement of HSC mobilization from the BM.[118] The proteasome inhibitor PS-341, currently used in MM therapy, blocks the growth of MM cells by decreasing their adherence to BMSCs and the related protection against drug-induced apoptosis.[119] Another strategy is the inhibition of TNF-α production by BM cells, with a monoclonal antibody against the extracellular domain of TNF-α, called infliximab. Two studies have investigated the use of infliximab in patients with low-risk MDS. In both reports, the drug showed a limited but significant activity and no particular side-effects.[120]

Another recently approved therapeutic approach involves inhibiting angiogenesis; several inhibitors of VEGF are currently used in the treatment of different hemopathies.[104, 121]

The concept behind most of these therapeutic approaches implies that to increase therapeutic efficacy it is necessary to use a strategy in which the seed (malignant HSCs) and the soil (altered BMM) must be targeted simultaneously.

Conclusions

This paper highlights the key data demonstrating that changes in the signals delivered by BM endosteal and/or perivascular niches may lead to an impairment of survival, differentiation and proliferation of HSCs. Thus, aberrant BM niches participating in HSC regulation contribute in a crucial way to the progression of hemopathies. Therefore, the molecular cues that contribute towards BM niches alteration during the onset and development of hemopathies represent a new challenging therapeutic target.

References

1. Zhong JF, Zhao Y, Sutton S, et al. Gene expression profile of murine long-term reconstituting vs. short-term reconstituting hematopoietic stem cells. Proc Natl Acad Sci USA 2005;102:2448-53.
2. Dexter TM, Wright EG, Krizsa F, Lajtha LG. Regulation of haemopoietic stem cell proliferation in long term bone marrow cultures. Biomedicine/[publiee pour l'AAICIG] 1977; 27:344-9.
3. Islam A, Catovsky D, Goldman JM, Galton DA. Histomorphological study of cellular interactions between stromal and haemopoietic stem cells in normal and leukaemic bone marrow. Histopathology 1984;8:293-313.
4. Lord BI, Testa NG, Hendry JH. The relative spatial distributions of CFUs and CFUc in the normal mouse femur. Blood 1975;46: 65-72.
5. Nilsson SK, Johnston HM, Coverdale JA. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. Blood 2001;97: 2293-9.
6. Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 1978; 4:7-25.
7. Kollet O, Dar A, Shivtiel S, et al. Osteoclasts degrade endosteal components and promote mobilization of hemato-poietic progenitor cells. Nat Med 2006;12:657-64.
8. Kubota Y, Takubo K, Suda T. Bone marrow long label-retaining cells reside in the sinusoidal hypoxic niche. Biochem Biophys Res Commun 2008;366:335-9.
9. Taichman RS, Reilly MJ, Emerson SG. Human osteoblasts support human hematopoietic progenitor cells in vitro bone marrow cultures. Blood 1996;87:518-24.
10. Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, et al. Identification of the hemato-poietic stem cell niche and control of the niche size. Nature 2003;425:836-41.
11. Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 2005;121:1109-21.
12. Watchman CJ, Bourke VA, Lyon JR, et al. Spatial distribution of blood vessels and CD34+ hematopoietic stem and progenitor cells within the marrow cavities of human cancellous bone. J Nucl Med 2007;48:645-54.
13. Avecilla ST, Hattori K, Heissig B, et al. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. Nat Med 2004;10:64-71.
14. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haemato-poietic stem cell niche. Nature 2003;425: 841-6.
15. Kiel MJ, Morrison SJ. Uncertainty in the niches that maintain haematopoietic stem cells. Nat Rev Immunol 2008;8:290-301.
16. Kopp HG, Avecilla ST, Hooper AT, Rafii S. The bone marrow vascular niche: home of HSC differentiation and mobilization. Physiology (Bethesda, Md.) 2005;20:349-56.
17. Rafii S, Mohle R, Shapiro F, et al. Regulation of hematopoiesis by microvascular endothelium. Leuk Lymphoma 1997; 27:375-86.
18. Wilson A, Oser GM, Jaworski M, et al. Dormant and self-renewing hematopoietic stem cells and their niches. Ann N Y Acad Sci 2007;1106:64-75.
19. Adams GB, Scadden DT. The hematopoietic stem cell in its place. Nat Immunol 2006;7:333-7.
20. Arai F, Hirao A, Suda T. Regulation of hematopoietic stem cells by the niche. Trends Cardiovasc Med 2005;15:75-9.
21. Yin T, Li L. The stem cell niches in bone. J Clin Invest 2006;116:1195-201.
22. Heissig B, Hattori K, Dias S, et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 2002;109:625-37.
23. Mohle R, Bautz F, Rafii S, et al. The chemokine receptor CXCR-4 is expressed on $CD34^+$ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood 1998;91:4523-30.
24. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity 2006;25:977-88.
25. Nilsson SK, Johnston HM, Whitty GA, et al. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood 2005;106:1232-9.
26. Papayannopoulou T, Priestley GV, Nakamoto B, et al. Molecular pathways in bone marrow homing: dominant role of alpha(4)beta(1) over beta(2)-integrins and selectins. Blood 2001;98:2403-11.
27. Katayama Y, Hidalgo A, Furie BC, et al. PSGL-1 participates in E-selectin-mediated progenitor homing to bone marrow: evidence for cooperation between E-selectin ligands and alpha4 integrin. Blood 2003; 102:2060-7.
28. Jung Y, Wang J, Song J, et al. Annexin II expressed by osteoblasts and endothelial cells regulates stem cell adhesion, homing, and engraftment following transplantation. Blood 2007;110:82-90.
29. Stier S, Ko Y, Forkert R, et al. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. J Exp Med 2005;201:1781-91.
30. Chanprasert S, Geddis AE, Barroga C, Fox NE, Kaushansky K. Thrombopoietin (TPO) induces c-myc expression through a PI3K- and MAPK-dependent pathway that is not mediated by Akt, PKCzeta or mTOR in TPO-dependent cell lines and primary megakaryocytes. Cell Signal 2006;18:1212-8.
31. Kaushansky K, Broudy VC, Grossmann A, et al. Thrombopoietin expands erythroid progenitors, increases red cell production, and enhances erythroid recovery after myelosuppressive therapy. J Clin Invest 1995;96:1683-7.
32. Nagahisa H, Nagata Y, Ohnuki T, et al. Bone marrow stromal cells produce thrombopoietin and stimulate megakaryocyte growth and maturation but suppress proplatelet formation. Blood 1996;87:1309-16.
33. Arai F, Hirao A, Ohmura M, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 2004;118:149-61.
34. Puri MC, Bernstein A. Requirement for the TIE family of receptor tyrosine kinases in adult but not fetal hematopoiesis. Proc Natl Acad Sci USA 2003;100:12753-8.
35. Duncan AW, Rattis FM, DiMascio LN, et al. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat Immunol 2005;6:314-22.
36. Maillard I, Koch U, Dumortier A, et al. Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells. Cell Stem Cell 2008;2:356-66.
37. Stier S, Cheng T, Dombkowski D, et al. Notch1 activation increases hematopoietic stem cell self-renewal *in vivo* and favors lymphoid over myeloid lineage outcome. Blood 2002;99:2369-78.
38. Lane WJ, Dias S, Hattori K, et al. Stromal-derived factor 1-induced megakaryocyte migration and platelet production is dependent on matrix metalloproteinases. Blood 2000;96: 4152-9.
39. Pui JC, Allman D, Xu L, et al. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. Immunity 1999;11:299-308.
40. de La Coste A, Six E, Fazilleau N, et al. In vivo and in absence of a thymus, the enforced expression of the Notch ligands delta-1 or delta-4 promotes T cell development with specific unique effects. J Immunol 2005;174:2730-7.
41. Jaleco AC, Neves H, Hooijberg E, et al. Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. J Exp Med 2001;194:991-1002.
42. Semerad CL, Christopher MJ, Liu F, et al. G-CSF potently inhibits osteoblast activity and CXCL12 mRNA expression in the bone marrow. Blood 2005;106:3020-7.
43. Petit I, Szyper-Kravitz M, Nagler A, et al. G-CSF induces stem cell mobilization by

decreasing bone marrow SDF-1 and up-regulating CXCR4. Nat Immunol 2002;3: 687-94.
44. Kiel MJ, Radice GL, Morrison SJ. Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. Cell stem cell 2007;1:204-17.
45. Scott LM, Priestley GV, Papayannopoulou T. Deletion of alpha4 integrins from adult hematopoietic cells reveals roles in homeostasis, regeneration, and homing. Mol Cell Biol 2003;23: 9349-60.
46. Yoshihara H, Arai F, Hosokawa K, et al. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell stem cell 2007;1:685-97.
47. Van Den Berg DJ, Sharma AK, Bruno E, Hoffman R. Role of members of the Wnt gene family in human hematopoiesis. Blood 1998;92:3189-202.
48. Willert K, Brown JD, Danenberg E, et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 2003;423:448-52.
49. Ma Q, Jones D, Borghesani PR, et al. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc Natl Acad Sci USA 1998;95:9448-53.
50. Kyle RA, Therneau TM, Rajkumar SV, et al. A long-term study of prognosis in monoclonal gammopathy of undetermined significance. N Engl J Med 2002;346:564-9.
51. Ma X, Does M, Raza A, Mayne ST. Myelo-dysplastic syndromes: incidence and survival in the United States. Cancer 2007;109:1536-42.
52. Walkley CR, Orkin SH. Rb is dispensable for self-renewal and multilineage differentiation of adult hematopoietic stem cells. Proc Natl Acad Sci USA 2006;103:9057-62.
53. Walkley CR, Shea JM, Sims NA, et al. Rb regulates interactions between hemato-poietic stem cells and their bone marrow microenvironment. Cell 2007;129: 1081-95.
54. Yilmaz OH, Valdez R, Theisen BK, et al. Pten dependence distinguishes haemato-poietic stem cells from leukaemia-initiating cells. Nature 2006; 441:475-82.
55. Zhang J, Grindley JC, Yin T, et al. PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature 2006;441:518-22.
56. Walkley CR, Olsen GH, Dworkin S, et al. A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency. Cell 2007;129: 1097-110.
57. Cortelezzi A, Fracchiolla NS, Mazzeo LM, et al. Endothelial precursors and mature endothelial cells are increased in the peripheral blood of myelodysplastic syndromes. Leuk Lymphoma 2005;46: 1345-51.
58. Fang B, Zheng C, Liao L, et al. Identification of human chronic myelogenous leukemia progenitor cells with hemangioblastic characteristics. Blood 2005;105:2733-40.
59. Zhang H, Vakil V, Braunstein M, et al. Circulating endothelial progenitor cells in multiple myeloma: implications and significance. Blood 2005;105:3286-94.
60. Rigolin GM, Fraulini C, Ciccone M, et al. Neoplastic circulating endothelial cells in multiple myeloma with 13q14 deletion. Blood 2006;107:2531-5.
61. Streubel B, Chott A, Huber D, et al. Lymphoma-specific genetic aberrations in microvascular endothelial cells in B-cell lymphomas. N Engl J Med 2004;351:250-9.
62. Wright EG. Ionizing radiation and leukaemia: more questions than answers. Hematol Oncol 2005;23:119-26.
63. Barcellos-Hoff MH, Park C, Wright EG. Radiation and the microenvironment - tumorigenesis and therapy. Nature Reviews 2005;5:867-75.
64. Ma J, Shi M, Li J, et al. Senescence-unrelated impediment of osteogenesis from Flk1+ bone marrow mesenchymal stem cells induced by total body irradiation and its contribution to long-term bone and hematopoietic injury. Haematologica 2007; 92:889-96.
65. Dilly SA, Jagger CJ. Bone marrow stromal cell changes in haematological malignancies. J Clin Pathol 1990;43:942-6.
66. Sudhoff T, Wehmeier A, Kliche KO, et al. Levels of circulating endothelial adhesion molecules (sE-selectin and sVCAM-1) in adult patients with acute leukemia. Leukemia 1996;10:682-6.
67. Veiga JP, Costa LF, Sallan SE, et al. Leukemia-stimulated bone marrow endothelium promotes leukemia cell survival. Exp Hematol 2006;34: 610-21.
68. Bouzin C, Brouet A, De Vriese J, et al. Effects of vascular endothelial growth factor on the lymphocyte-endothelium interactions: identification of caveolin-1 and nitric oxide as control points of endothelial cell anergy. J Immunol 2007;178:1505-11.
69. Jo DY, Hwang JH, Kim JM, et al. Human bone marrow endothelial cells elaborate non-stromal-cell-derived factor-1 (SDF-1)-dependent chemoattraction and SDF-1-dependent transmigration of haematopoietic progenitors. Br J Haematol 2003;121: 649-52.
70. Peled A, Grabovsky V, Habler L, et al. The chemokine SDF-1 stimulates integrin-mediated arrest of CD34$^+$ cells on vascular endothelium under shear flow. J Clin Invest 1999; 104:1199-211.
71. Sipkins DA, Wei X, Wu JW, et al. In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature 2005;435:969-73.
72. Bradstock KF, Makrynikola V, Bianchi A, et al. Effects of the chemokine stromal cell-derived factor-1 on the migration and localization of precursor-B acute lymphoblastic leukemia cells within bone marrow stromal layers. Leukemia 2000; 14:882-8.
73. Shen W, Bendall LJ, Gottlieb DJ, Bradstock KF. The chemokine receptor CXCR4 enhances integrin-mediated in vitro adhesion and facilitates engraftment of leukemic precursor-B cells in the bone marrow. Exp Hematol 2001;29:1439-47.
74. Hazlehurst LA, Landowski TH, Dalton WS. Role of the tumor microenvironment in mediating de novo resistance to drugs and physiological mediators of cell death. Oncogene 2003;22:7396-402.
75. Matsunaga T, Takemoto N, Sato T, et al. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. Nat Med 2003;9: 1158-65.
76. De Toni F, Racaud-Sultan C, Chicanne G, et al. A crosstalk between the Wnt and the adhesion-dependent signaling pathways governs the chemosensitivity of acute myeloid leukemia. Oncogene 2006;25: 3113-22.
77. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood 1999; 93:1658-67.
78. Damiano JS, Hazlehurst LA, Dalton WS. Cell adhesion-mediated drug resistance (CAM-DR) protects the K562 chronic myelogenous leukemia cell line from apoptosis induced by BCR/ABL inhibition, cytotoxic drugs, and gamma-irradiation. Leukemia 2001;15: 1232-9.
79. Hazlehurst LA, Dalton WS. Mechanisms associated with cell adhesion mediated drug resistance (CAM-DR) in hematopoietic malignancies. Cancer Metastasis Rev 2001;20:43-50.
80. Mudry RE, Fortney JE, York T, et al. Stromal cells regulate survival of B-lineage leukemic cells during chemotherapy. Blood 2000;96:1926-32.
81. Narendran A, Ganjavi H, Morson N, et al. Mutant p53 in bone marrow stromal cells increases VEGF expression and supports leukemia cell growth. Exp Hematol 2003;31:693-701.
82. Nefedova Y, Cheng P, Alsina M, et al. Involvement of Notch-1 signaling in bone marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines. Blood 2004;103: 3503-10.
83. Andreasson B, Swolin B, Kutti J. Patients with idiopathic myelofibrosis show increased CD34$^+$ cell concentrations in

peripheral blood compared to patients with polycythaemia vera and essential thrombocythaemia. Eur J Haematol 2002;68: 189-93.
84. Barosi G, Viarengo G, Pecci A, et al. Diagnostic and clinical relevance of the number of circulating CD34+ cells in myelofibrosis with myeloid metaplasia. Blood 2001;98:3249-55.
85. Bruns I, Czibere A, Fischer JC, et al. The hematopoietic stem cell in chronic phase CML is characterized by a transcriptional profile resembling normal myeloid progenitor cells and reflecting loss of quiescence. Leukemia 2009 Jan 22 [Epub Ahead of Print].
86. Ries C, Loher F, Zang C, et al. Matrix metalloproteinase production by bone marrow mononuclear cells from normal individuals and patients with acute and chronic myeloid leukemia or myelodysplastic syndromes. Clin Cancer Res 1999;5:1115-24.
87. Barille S, Akhoundi C, Collette M, et al. Metalloproteinases in multiple myeloma: production of matrix metalloproteinase-9 (MMP-9), activation of proMMP-2, and induction of MMP-1 by myeloma cells. Blood 1997;90:1649-55.
88. Janowska-Wieczorek A, Marquez LA, Matsuzaki A, et al. Expression of matrix metalloproteinases (MMP-2 and -9) and tissue inhibitors of metalloproteinases (TIMP-1 and -2) in acute myelogenous leukaemia blasts: comparison with normal bone marrow cells. Br J Haematol 1999; 105:402-11.
89. Xu M, Bruno E, Chao J, et al. Constitutive mobilization of CD34+ cells into the peripheral blood in idiopathic myelofibrosis may be due to the action of a number of proteases. Blood 2005;105:4508-15.
90. Brouty-Boye D, Briard D, Azzarone B, et al. Effects of human fibroblasts from myelometaplasic and non-myelometaplasic hematopoietic tissues on CD34+ stem cells. Int J Canc 2001;92:484-8.
91. Cervi D, Shaked Y, Haeri M, et al. Enhanced natural-killer cell and erythropoietic activities in VEGF-A-overexpressing mice delay F-MuLV-induced erythroleukemia. Blood 2007;109:2139-46.
92. Koistinen P, Siitonen T, Mantymaa P, et al. Regulation of the acute myeloid leukemia cell line OCI/AML-2 by endothelial nitric oxide synthase under the control of a vascular endothelial growth factor signaling system. Leukemia 2001;15:1433-41.
93. Hatfield K, Ryningen A, Corbascio M, Bruserud O. Microvascular endothelial cells increase proliferation and inhibit apoptosis of native human acute myelogenous leukemia blasts. Int J Canc 2006;119: 2313-21.
94. Wang L, O'Leary H, Fortney J, Gibson LF. Ph+/VE-cadherin+ identifies a stem cell like population of acute lymphoblastic leukemia sustained by bone marrow niche cells. Blood 2007;110:3334-44.
95. Chatterjee M, Honemann D, Lentzsch S, et al. In the presence of bone marrow stromal cells human multiple myeloma cells become independent of the IL-6/gp130/ STAT3 pathway. Blood 2002;100:3311-8.
96. Folkman J. Role of angiogenesis in tumor growth and metastasis. Semin Oncol 2002;29:15-8.
97. Hussong JW, Rodgers GM, Shami PJ. Evidence of increased angiogenesis in patients with acute myeloid leukemia. Blood 2000;95:309-13.
98. AR, Kay NE, Peterson LC. Increased bone marrow angiogenesis in B cell chronic lymphocytic leukemia. Leukemia 2000;14: 1414-8.
99. Lundberg LG, Lerner R, Sundelin P, et al. Bone marrow in polycythemia vera, chronic myelocytic leukemia, and myelofibrosis has an increased vascularity. Am J Pathol 2000;157: 15-9.
100. Padro T, Ruiz S, Bieker R, Burger H, Steins M, Kienast J, et al. Increased angiogenesis in the bone marrow of patients with acute myeloid leukemia. Blood 2000;95:2637-44.
101. Vacca A, Ribatti D, Roncali L, et al. Bone marrow angiogenesis and progression in multiple myeloma. Br J Haematol 1994;87: 503-8.
102. Fragoso R, Elias AP, Dias S. Autocrine VEGF loops, signaling pathways, and acute leukemia regulation. Leuk Lymphoma 2007;48:481-8.
103. Kelaidi C, Eclache V, Fenaux P. The role of lenalidomide in the management of myelodysplasia with del 5q. Br J Haematol 2008;140:267-78.
104. Kumar S, Witzig TE, Dispenzieri A, et al. Effect of thalidomide therapy on bone marrow angiogenesis in multiple myeloma. Leukemia 2004;18:624-7.
105. Ribatti D, Vacca A, Dammacco F, English D. Angiogenesis and anti-angiogenesis in hematological malignancies. J. Hematother Stem Cell Res 200312:11-22.
106. Dias S, Choy M, Alitalo K, Rafii S. Vascular endothelial growth factor (VEGF)-C signaling through FLT-4 (VEGFR-3) mediates leukemic cell proliferation, survival, and resistance to chemotherapy. Blood 2002; 99:2179-84.
107. Rafii S, Shapiro F, Pettengell R, et al. Human bone marrow microvascular endothelial cells support long-term proliferation and differentiation of myeloid and megakaryocytic progenitors. Blood 1995;86:3353-63.
108. Nefedova Y, Landowski TH, Dalton WS. Bone marrow stromal-derived soluble factors and direct cell contact contribute to de novo drug resistance of myeloma cells by distinct mechanisms. Leukemia 2003;17: 1175-82.
109. Hertenstein B, Hambach L, Bacigalupo A, et al. Development of leukemia in donor cells after allogeneic stem cell transplantation--a survey of the European Group for Blood and Marrow Transplantation (EBMT). Haematologica 2005;90:969-75.
110. Kai T, Spradling A. An empty Drosophila stem cell niche reactivates the proliferation of ectopic cells. Proc Natl Acad Sci USA 2003;100:4633-8.
111. Kai T, Spradling A. Differentiating germ cells can revert into functional stem cells in Drosophila melanogaster ovaries. Nature 2004;428:564-9.
112. Ballen KK, Shpall EJ, Avigan D, et al. Phase I trial of parathyroid hormone to facilitate stem cell mobilization. Biol Blood Marrow Transplant 2007;13:838-43.
113. Napoli C, William-Ignarro S, Byrns R, et al. Therapeutic targeting of the stem cell niche in experimental hindlimb ischemia. Nat Clin Pract 2008;5:571-9.
114. Adams GB, Martin RP, Alley IR, et al. Therapeutic targeting of a stem cell niche. Nature Biotechnol 2007;25:238-43.
115. Broxmeyer HE, Orschell CM, Clapp DW, et al. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. J Exp Med 2005;201:1307-18.
116. Cashen AF, Link D, Devine S, DiPersio J. Cytokines and stem cell mobilization for autologous and allogeneic transplantation. Curr Hematol Rep 2004;3:406-12.
117. Levesque JP, Hendy J, Takamatsu Y, et al. Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. J Clin Invest 2003;111: 187-96.
118. Calandra G, McCarty J, McGuirk J, et al. AMD3100 plus G-CSF can successfully mobilize CD34+ cells from non-Hodgkin's lymphoma, Hodgkin's disease and multiple myeloma patients previously failing mobilization with chemotherapy and/or cytokine treatment: compassionate use data. Bone Marrow Transplant 2008;41: 331-8.
119. Hideshima T, Richardson P, Chauhan D, et al. The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. Cancer Res 2001;61: 3071-6.
120. Maciejewski JP, Risitano AM, Sloand EM, et al. A pilot study of the recombinant soluble human tumour necrosis factor receptor (p75)-Fc fusion protein in patients with myelodysplastic syndrome. Br J Haematol 2002;117:119-26.
121. Klasa RJ, List AF, Cheson BD. Rational approaches to design of therapeutics targeting molecular markers. Hematology Am Soc Hematol Educ Program 2001:443-62.